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MECHANISMS OF NANOPARTICLE FORMATION AND GENE DELIVERY OF POLYPLEX-BASED DELIVERY SYSTEMS

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DOCTORAL DISSERTATION

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Abstract

Gene therapy provides a promising option for treatment of various diseases, but the fact remains that the large number of gene delivery systems has met with little therapeutic success. Viral gene delivery has a high degree of specificity and efficacy, but it does not provide sufficient safety for clinical applications. Therefore, the search for an efficient alternative, a synthetic gene delivery vehicle (vector), has been active.

Typically, non-viral delivery vectors are based on the use of cationic polymers which bind and compact DNA via electrostatic interactions into nanoparticles (polyplexes). The ability of a cationic polymer to bind and condense DNA is important for effective delivery because good packing not only protects DNA against degradation in the extracellular space, but also allows effective release of DNA inside cells. While cationic polymers are relatively nontoxic and safe, they lack significant efficacy. This major drawback of non-viral vectors is largely due to a poor understanding of the mechanism underlying the complexation and gene delivery process. Furthermore, the lack of reliable methods to study the binding between DNA and cationic polymers has hindered development in synthetic gene delivery systems.

The aim of this study was to investigate the mechanisms of DNA complex formation and gene transfer mediated by cationic polymers with different structures (poly-L-lysine, PLL; polyethylenimines, PEIs; poly-β-amino esters, PBAEs) and transfection efficiencies.

Firstly, this thesis combines time-resolved fluorescence spectroscopy, a novel method developed in our laboratory, with cell transfection studies in order to elucidate how polymer structure can affect DNA binding and influence gene delivery outcomes. This method allows the quantitative determination of polymer–DNA interaction and binding. We showed that the mechanism of PEI–DNA and PLL–DNA complex formation was positively cooperative with a saturation limit near 100% at a polymer/DNA molar (N/P) ratio of 2, whereas most of PBAE–DNA complexes expressed negative cooperativity and reached a saturation level close to 80%. The polymer topology, the type of amines (primary, secondary and tertiary) and their density, and the environmental pH had a clear effect on the binding constants and the degree of cooperativity. The possible correlation between fluorescence parameters and transfection efficiency was investigated with a series of PBAEs. Their transfection efficiency showed an increasing trend in association with the relative efficiency of PBAE–DNA nanoparticle formation.

Secondly, the role of free polymer in polyplex formation and gene delivery was examined with PEI as a model vector. For PEI polyplexes, the formation of the polyplex core was completed at N/P ~2 and the excess of polymer formed a protective shell around the core. Unlike PLL, PEI molecules were able to undergo an exchange between the core and shell of the polyplexes. Such differences in structural dynamics of these polyplexes may partly provide an explanation for the differences seen in their DNA release and transfection efficacy at the cellular level. The excess of PEI in the shell had no effect on the physical state of polyplexes, suggesting that the polyplex core retains its original structure during shell formation. However, the excess of PEI

was a crucial factor in successful transfection. The role of free PEI in the gene transfection process was examined in cell cultures with modified cell-surface glycosaminoglycans (GAGs) content. This study showed that free PEI is essential for minimizing the undesirable binding of polyplexes to cell-surface glycosaminoglycans, which may otherwise pose a barrier in non-viral gene delivery.

Lastly, we focused on the role of PEI structure in PEI–liposome–DNA delivery systems (lipopolyplexes). We found that the enhancement of lipopolyplex-mediated delivery by different types of PEI species is common and associated with PEI size rather than structure.

In conclusion, the present study demonstrated that the fluorescence spectroscopy approach for the analysis of gene delivery systems can provide valuable quantitative information about the binding behaviour of various cationic polymers to DNA. The improved understanding of mechanisms behind formation of these complexes can contribute to the design of polymeric delivery vectors with improved properties. Furthermore, this study sheds light on the mechanisms by which free polymer enhances gene transfer. It explains why high N/P ratios are needed for effective transfection and how the interactions between free polymer and cell-surface GAGs lead to alterations in gene transfer by the polyplexes.

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Helsinki, October 2019

Martina Hanzlíková

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List of original publications

This thesis is based on the following publications:

I. Ketola TM, **Hanzlíková M**, Urtti A, Lemmetyinen H, Yliperttula M, Vuorimaa E, 2011. Role of polyplex intermediate species on gene transfer efficiency: Polyethylenimine-DNA complexes and time-resolved fluorescence spectroscopy. Journal of Physical Chemistry B, 115, 1895-1902.

II. Vuorimaa E, Ketola TM, Green JJ, **Hanzlíková M**, Lemmetyinen H, Langer R, Anderson DG, Urtti A, Yliperttula M, 2011. Poly(β-amino ester)-DNA complexes: Time-resolved fluorescence and cellular transfection studies. Journal of Controlled Release, 154, 171-176.

III. Ketola TM, **Hanzlíková M**, Leppänen L, Raviña M, Bishop CJ, Green JJ, Urtti A, Lemmetyinen H, Yliperttula M, Vuorimaa-Laukkanen E, 2013. Independent versus cooperative binding in polyethylenimine-DNA and poly(l-lysine)-DNA polyplexes. Journal of Physical Chemistry B, 117, 10405-10413.

IV. Vuorimaa-Laukkanen E, Lisitsyna ES, Ketola TM, Morin-Pickardat E, Liang H, **Hanzlíková M**, Yliperttula M, 2017. Difference in the core-shell dynamics of polyethyleneimine and poly(L-lysine) DNA polyplexes. European Journal of Pharmaceutical Sciences, 103, 122-127.

V. **Hanzlíková M**, Ruponen M, Galli E, Raasmaja A, Aseyev V, Tenhu H, Urtti A, Yliperttula M, 2011. Mechanisms of polyethylenimine-mediated DNA delivery: Free carrier helps to overcome the barrier of cell-surface glycosaminoglycans. Journal of Gene Medicine, 13, 402-409.

VI. **Hanzlíková M**, Soininen P, Lampela P, Männistö PT, Raasmaja A, 2009. The role of PEI structure and size in the PEI/liposome-mediated synergism of gene transfection. Plasmid, 61, 15-21.

The publications are referred to in the text by their Roman numerals and have been reprinted here with the kind permission of their copyright holders.

Abbreviations

$(K_{co})^{\alpha}$	overall cooperative binding constant
ARPE-19	human retinal pigment epithelial cell line
α	experimental Hill coefficient
B	proportion of DNA bound by the polymer
bp	base pairs
BPEI	branched polyethylenimine
β-gal	beta-galactosidase
CHO	Chinese hamster ovary cell line
CpG	guanosine-cytosine monomer
CS	chondroitin sulfate
CV1-P	monkey kidney fibroblasts
Cy3	cyanine dye
DAS	decay-associated spectra
DC-Chol	3β-[N-(dimethylaminoethane)carbamoyl]cholesterol
DMRIE	1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium bromide
DNA	deoxyribonucleic acid
DOCSPER	1,3-dioleoyloxy-2-(N5-carbamoyl-spermine)propane
DOGS	dioctadecylamidoglycylspermine
DOPE	1,2-dioleyl phosphatidylethanolamine
DOSPA	2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-*N,N*-dimethyl-1-propanaminium trifluoroacetate
DOSPER	1,3-dioleoxy-2-(6-carboxy-spermyl)propylamide
DOTAP	1,2-dioleoyloxy-3-(trimethylammonio)propane
DOTMA	*N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride
ETI	ethidium bromide
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FRET	fluorescence resonance energy transfer
GAG	glycosaminoglycan
$GalTI^{-}$	Chinese hamster ovary cells devoid of galactosyltransferase I activity
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HS	heparan sulfate
$HSPol^{-}$	Chinese hamster ovary cells devoid of heparin sulfate polymerase activity
K_{co}	average cooperative binding constant per amine
LDH	lactate dehydrogenase
LPEI	linear polyethylenimine

LPP	lipopolyplex
λ	wavelength
MES	2-N-morpholino ethane sulfonic acid
mRNA	messenger ribonucleic acid
N/P ratio	nitrogen to phosphate ratio; molar ratio of polycation protonable amines to DNA phosphates
NAD^+	nicotinamide adenine dinucleotide
NLS	nuclear localization signal
NMR	nuclear magnetic resonance
NPC	nuclear pore complex
ODN	oligodeoxynucleotide
ONPG	*o*-nitrophenyl β-D-galactopyranoside
PBAE	poly(beta-amino ester)
PCR	polymerase chain reaction
pDNA	plasmid DNA
PEG	poly(ethylene glycol)
PEI	polyethylenimine
pK_a	logarithmic acid dissociation constant
PLL	poly-L-lysine
qRT-PCR	quantitative real-time polymerase chain reaction
RES	reticuloendothelial system
R_g/R_h	ratio of radius of gyration and hydrodynamic radius
RGD	arginine-glycine-aspartic acid
RLU	relative light unit
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
siRNA	small interfering ribonucleic acid
SMC	smooth muscle cells
SNARE	N-ethylmaleimide- sensitive factor adaptor protein receptor
SPEI	small PEI, branched 800 Da polyethylenimine
TCSPC	time-correlated single photon counting
τ	fluorescence lifetime
w/w	weight to weight ratio
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

1 Introduction

Gene therapy has enormous potential to provide a new treatment for incurable diseases. The basic concept of gene therapy involves transferring corrective genetic material into a dysfunctional patient's cells that will by their own mechanisms produce (or silence) the therapeutic protein, which in turn will cure or slow down the progression of disease (Verma and Somia, 1997).

There are different types of nucleic acid-based therapeutic strategies available: DNA-mediated therapy, antisense therapy, ribozymes, and RNA interference (RNAi). Currently, all of these are focused on somatic cells, and no strategy targets the germ line. DNA-mediated therapy utilizes plasmid DNA (pDNA) to turn on or restore function of endogenous gene, resulting in expression of the therapeutic protein (gain-of-function) (Friedmann and Roblin, 1972). In antisense therapy, antisense oligodeoxyribonucleotides complementarily bind to a mRNA sequence of a disease-causing gene and turn off the production of the protein (loss-of-function) (Hughes et al., 2001). The gene expression of unwanted protein can be selectively down-regulated also by cleaving mRNA in a sequence-specific manner using ribozymes (Lewin and Hauswirth, 2001) or by a RNAi-based strategy (Bumcrot et al., 2006).

The gene therapy was originally aimed as a treatment for inherited monogenic diseases such as X-linked severe combined immunodeficiency (SCID-X1), haemophilia, muscular dystrophy, hypercholesterolemia, or cystic fibrosis. Today, with the information available about human disease-associated genes, gene-based therapeutics are being developed also for acquired diseases, like cancer, neurodegenerative disorders, cardiovascular diseases, and infectious diseases, or as therapeutic DNA vaccines. Despite advances in the field, exciting prospects, and the simple concept, gene therapy has met with variable success (Cavazzana-Calvo, 2000) and is still far from a widespread therapeutic reality. Since the first approved clinical trial with a therapeutic gene in humans for a very rare adenosine deaminase severe combined immunodeficiency (ADA-SCID) syndrome in 1990 (Blaese, 1995), more than two thousand clinical studies have been conducted worldwide. At the time of writing this thesis, however, only four gene therapy products have been granted marketing approval. The first two products, Gencidine (in 2003) and Oncorine (in 2005), are available in China to treat head and neck cancer. The European Medicines Agency (EMA) approved Glybera (in 2012) aimed at patients with rare lipoprotein lipase deficiency and Strimvelis (in 2016) designed as a paediatric gene therapy for ADA-SCID.

The major challenge for effective gene therapy is the issue of delivery (Verma, 1997). Nucleic-acid therapeutics need to enter cells in order to produce the therapeutic protein. However, nucleic acids (e.g. pDNA, ODNs, siRNA) have poor stability in the systemic circulation, limited cell membrane permeability due to their hydrophilicity and negative charge, and therefore, cannot be delivered by conventional means. They require delivery vehicles (i.e. carriers or vectors) in order to reach their target sites intact. Different strategies using physical methods or synthetic materials as vectors have been developed to protect the nucleic acid from degradation and facilitate its

delivery to target cells or organs. The important requirements of good gene therapy vector are high efficiency of targeted delivery and expression without inducing harmful side effects (Thomas et al., 2003). Ideally, the vector should be also non-immunogenic, non-toxic, biodegradable, stable in physiological conditions, resistant to enzymatic degradation, and able to efficiently release nucleic acids inside the cells of interest. Furthermore, vectors should be safe and easy to produce, inexpensive, and simple to administer to facilitate clinical use (Somia and Verma, 2000).

Current gene delivery vectors can be divided into two categories: viral and non-viral. The viral delivery involves replication-defective viruses that use highly evolved natural mechanisms to survive in the extracellular environment, to cross cellular membranes, and to shuttle their nucleic acids into the cytosol or nucleus with high efficiency. The main classes of viral vectors used in clinical studies are derived from retroviruses, lentiviruses, herpes simplex-1 viruses, adenoviruses, and adeno-associated viruses. Viral vectors facilitate the highest transfection rates among delivery systems, but the fact remains that they carry serious safety risks along with benefits. To avoid problems associated with their immunogenicity (Marshall, 1999) and insertional mutagenesis (Hacein-Bey-Abina et al., 2003), non-viral vectors have been employed as a safer alternative. Although the majority of gene delivery studies still rely on viruses, non-viral vectors have gained in popularity in recent years (Figure 1.1).

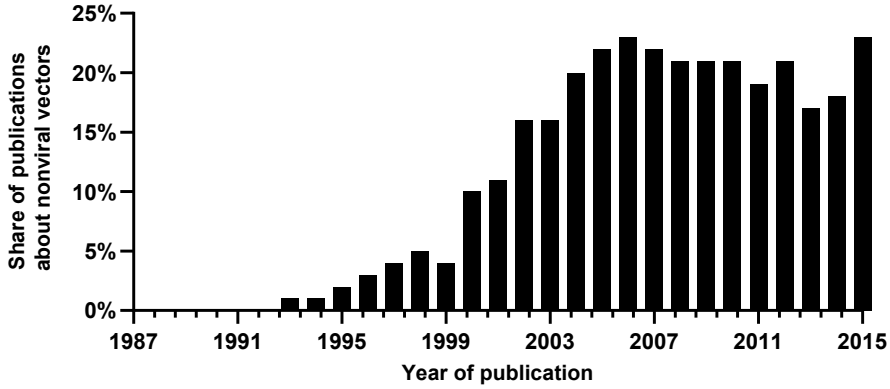


Figure 1.1 *Share of publications dealing with non-viral vectors[1] in the literature published about gene delivery vectors (viral and non-viral)[2] since the first introduction of cationic lipids and polymers as transfection vectors in 1987 (Felgner et al., 1987, Wu and Wu, 1987). Source: Scopus®, Elsevier B.V. Search terms: [1]("non-viral" or "nonviral") and "vector", [2]"viral" and "vector", as of October 2015.*

Commonly used non-viral vectors are based on cationic lipids, cationic polymers, and their polysaccharide conjugates. In addition to carrier-based methods, physical strategies for naked DNA delivery have been effective in some specific applications. Compared with viral vectors, non-viral vectors have relatively lower immunogenicity,

no risk of chromosomal insertion and can carry large-sized DNA. They can also address better the pharmaceutical industry than their viral counterparts because of a flexible design, greater control over their molecular composition during production, storage stability, and quality control. In spite of these advantages, a key limitation of the present generation of non-viral vectors is their poor transfection efficiency.

The main aim of this thesis was to study cationic polymers and mechanisms behind nanoparticle formation and cellular delivery. Better understanding of non-viral vector physiochemical properties regarding extracellular and intracellular delivery mechanisms is needed to overcome the problems associated with their low transfection efficacy.

2 Review of the literature

2.1 Barriers to effective non-viral gene delivery

Nucleic acid delivery from the moment of administration until uptake into the final target cells is a multistep process during which nucleic acid therapeutics must overcome numerous biological and physical barriers and also retain biological activity. Depending on the site of administration, they must cross: (i) extracellular barriers posed by blood vessels, blood proteins and cells, extracellular matrix, immune cells, or non-target cells, (ii) cell membrane, and (iii) multiple intracellular barriers (Figure 2.1).

Unprotected (naked) nucleic acids are unstable under physiological conditions and are very rapidly degraded by endogenous nucleases; the half-life of uncomplexed pDNA has been reported to be as short as 10 min in blood circulation (Kawabata et al. 1995) and 1–2 h in cytoplasm (Lechardeur et al., 1999). Therefore, a prerequisite for successful delivery is a gene carrier (vector) that fulfils protective and delivery function in extracellular and intracellular space. The ineffectiveness of the vector at any stage of the transfection process can substantially decrease gene delivery (Nguyen and Szoka, 2012).

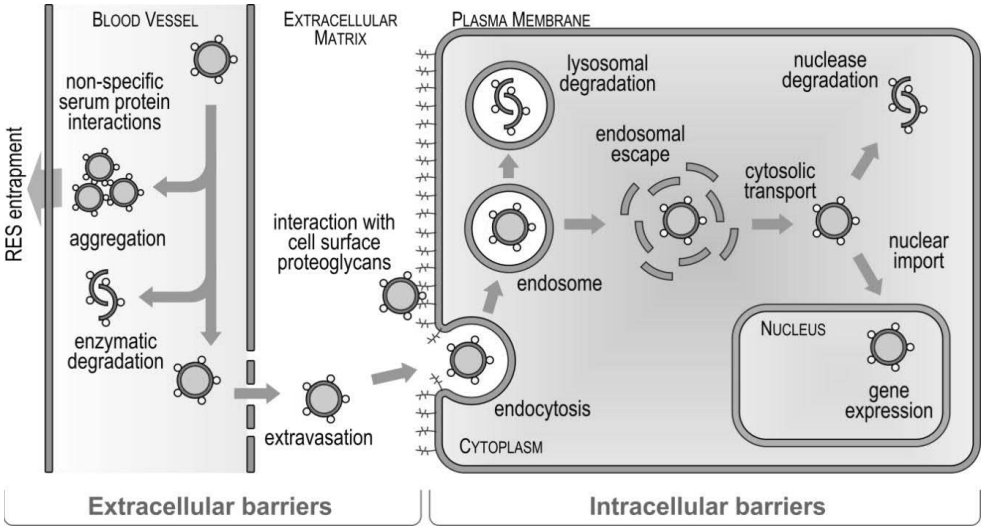


Figure 2.1 *Biological barriers to non-viral gene delivery vectors.*

2.1.1 Physical barriers – nanoparticle formulation and stability

The first challenge in the gene delivery process is the preparation of an effective vector–nucleic acid formulation that is well-characterized, specific, and stable in the extracellular environment. The most commonly used non-viral vectors, i.e. cationic lipids and polymers, electrostatically interact with polyanionic nucleic acids, resulting in a formation of submicron-sized particles – lipoplexes and polyplexes. Such complexes have unique particle characteristics that are influenced by various factors: (i) vector structure (e.g. molecular weight, branching, charge density, stability), (ii) ratio of positively charged vector to negatively charged nucleic acid, and (iii) formulation protocol (concentrations, speed and sequence of mixing, buffer components, complexation time) (Kircheis et al., 2001b). Altering these factors can dramatically change particle size and surface charge, and hence, strongly affect in vitro and in vivo gene delivery efficacy (van Gaal et al., 2011).

For instance, the particle net charge strongly affects the stability of polyplexes. At near neutrality charge ratio, polyplexes quickly form large aggregates. In contrast, positively charged polyplexes are stabilized in aqueous solutions by a hydrophilic cationic shell around the polyplex. On the one hand the increase in positive charges improves the solubility. On the other hand, it gives rise to free carrier that co-exists with complexes and is known to cause cellular toxicity (De Smedt et al., 2000). Even though positively charged polyplexes typically remain in solution, they become unstable and have a strong tendency to aggregate over time (Anchordoquy et al., 2001). The aggregation is more pronounced at physiological salt concentrations than in a low ionic environment and leads to the complete loss of polyplex bioactivity.

The size of nucleic acid–vector complexes and potential aggregation play key roles in determining the tissue distribution along with cellular uptake and intracellular processing (Rejman et al., 2004). A small compact delivery complex is important as the aggregated formulations can artificially elevate transfection rates in cell cultures (Xu and Anchordoquy, 2011), prevent passage through various biological barriers, and non-specifically accumulate in organs with fine capillary beds in the lungs, skin or intestine. The physical instability of complexes in solution is often connected to low storage stability, difficulties in reproducing biodistribution pattern, and with batch-to-batch variability in large-scale production (Anchordoquy and Koe, 2000).

2.1.2 Extracellular delivery barriers – reaching the target site

The significance of extracellular barriers is influenced by the route of administration. Nucleic acid therapeutics have been typically delivered locally (via direct injection to tumours, retina, or the central nervous system, or via inhalation to the lungs) and systemically via intravenous injection. Following the systemic delivery, the first barrier encountered by nucleic acid–vector complexes is the bloodstream. In blood circulation, positively charged complexes readily interact with abundant serum proteins, enzymes, blood cells, or may even adsorb to the blood vessel wall. Various proteins, lipids and other biomolecules quickly absorbs to the particle surface leading

to formation of a highly dynamic shell, known as the protein corona (Cedervall et al., 2007). This corona is difficult to control as its composition continuously changes at each stage of delivery and is dependent on both the vector chemistry and the initial physiological environment (Pozzi et al., 2015). A study by Capriotti et al. (2011) showed that more than two hundred different human plasma proteins such as apolipoproteins, complement system proteins, immunoglobulins, acute-phase proteins, coagulation-related proteins, and cell-adhesion proteins, bind to the surface of lipoplexes. This layer of biomolecules modifies physicochemical properties of vector complexes and facilitates their aggregation, contributes to changes in circulation half-time and biodistribution profile, affects toxicity and limits active targeting (Monopoli et al., 2012, Salvati et al., 2013).

The interaction with blood proteins evokes an innate immune response by initiating the alternative pathway of the complement system. Complement activation leads to opsonization of complexes, in particular with the C3b fragment, which is followed by rapid clearance in macrophages of the reticuloendothelial system (RES), causing the majority of complexes to end up in RES organs like the liver, spleen, and bone marrow (Plank et al., 1996). Furthermore, the excessive complement activation has been shown to contribute to severe inflammation and coagulation in an animal model (Merkel et al., 2011). Also pDNA sequences with unmethylated CpG motifs have been shown to promote strong inflammatory responses (Zhao et al., 2004).

One of the widely employed strategy to diminish the nonspecific interactions of delivery complexes with blood components is the shielding of positive charges of complexes with hydrophilic moieties, such as polyethylene glycol (PEG). PEGylated complexes have demonstrated reduced aggregation and enhanced colloidal stability in serum (Maurstad et al., 2013). PEGylation significantly prolongs circulation time of complexes in the blood stream, mainly by sterically hindering the adsorption of opsonizing serum proteins, resulting in reduced recognition and clearance by RES (Ogris et al., 1999, Finsinger et al., 2000, Petersen et al., 2002b). Although the PEGylation improves complex properties for systemic delivery, it can also prevent complexes from interactions with target cell membranes and endosomal membranes, thereby reducing a transfection potency of delivery systems (Hatakeyama et al., 2011, Pozzi et al., 2014). To solve this problem associated with the PEGylation, known as “PEG dilemma”, various methods for controlled deshielding of the PEG chains from the surface of complexes have been developed, leading to a stable PEG coating for overcoming extracellular barriers and to triggered PEG release in the intracellular space (Wang et al., 2012).

Unless administered locally, delivery complexes have to penetrate blood vessels in order to access interstitial space of target tissue. Extravasation through the capillary wall presents another delivery barrier, as the vascular permeability for complexes is limited. Under normal conditions, complexes may pass through blood capillaries only in the tissues of the RES organs, such as the liver, due to the presence of a discontinuous endothelium, which has fenestrations of up to 100 nm (Liu et al., 1992). However, many solid tumours exhibit defective blood vessels, which enables increased accumulation of complexes via the vascular phenomenon of an enhanced permeability and retention (EPR) effect (Seymour, 1992). Although many complexes

uses the EPR effect for passive tumour targeting, the targeting efficacy remains controversial (Prabhakar et al., 2013) and relatively modest compared with normal organs (Nakamura et al., 2016).

Moreover, the colloidal stability of delivery complexes is challenged by the significant ionic strength of plasma, which can weaken the interactions between the polycation and DNA (Wiethoff and Middaugh, 2003). Electrostatic interactions with negatively charged serum proteins such as albumin, and extracellular matrix components particularly glycoproteins, can lead to competitive binding and destabilization of polycation complexes, which can eventually cause partial or complete complex disintegration and premature release of nucleic acids from the vector (Nishikawa and Huang, 2001).

2.1.3 Cellular and intracellular delivery barriers

Once the complexes diffuse through the extracellular matrix to the target cells, they must cross a series of cellular barriers between the nucleus and extracellular space. These include the plasma membrane, the endosomal membrane, and the nuclear envelope. The plasma membrane is typically impermeable to large, hydrophilic and charged molecules, such as pDNA or non-viral vector complex, and thus, most of the non-viral vectors enter the cells via endocytosis. Beside the limited passage, plasma membrane has a very high content of anionic glycosylated membrane proteins that may alter both the cellular uptake and the intracellular trafficking of gene delivery complexes (Ruponen et al., 2003).

After internalization into membrane-bound endosomes, the non-viral vector must escape the endo-lysosomal pathway at an early stage, prior to the eventual enzymatic degradation. The endosomal escape is widely regarded as the major bottleneck in efficient gene delivery (Wang et al., 2012). The released complexes have their final target destination within the cytosol (e.g. ODNs, mRNA, siRNA) or are further trafficked through the cytosol to the nucleus (pDNA). The viscosity of the cytosol and the dense network of the cytoskeleton, together with the presence of cytosolic nucleases, pose another barrier before reaching the nuclear envelope (Lechardeur et al., 1999, Lukacs et al., 2000, Pollard et al., 2001).

The trafficking of pDNA across the nuclear membrane has been shown to proceed either through nuclear pores or as passive import during mitosis when the nuclear envelope temporarily breaks down and reforms (Zhou et al., 2004). Several early microinjection studies (Capecchi, 1980, Zabner et al., 1995, Pollard et al., 1998, Ludtke et al., 2002) demonstrated that the nuclear import of pDNA is relatively ineffective, and a considerable amount of pDNA entering the cytoplasm never reaches the nucleus. In crossing the intracellular barriers, non-viral vectors are significantly less efficient than their viral counterparts, which have an innate ability to internalize into host cells. It has been shown that only a small fraction of the DNA delivered by non-viral vector that is taken up by the cells is also expressed (Tachibana et al., 2002). The lower transfection efficiency of non-viral vectors arises from an inefficient intranuclear release of pDNA for transcription (Hama et al., 2006).

2.2 Non-viral gene delivery methods

2.2.1 Naked DNA-based methods

Needle injection – Naked pDNA injection into a local tissue or systemic circulation is the simplest delivery method. Initially, intramuscular injection of pDNA was found to induce gene expression (Wolff et al., 1990), but due to relatively low efficiency its main use remains in DNA-based immunization (Ulmer et al., 1993). In vivo gene expression was reported after direct injection to the liver (Hickman et al., 1994), lung airways (Meyer et al., 1995), or solid tumours (Yang and Huang, 1996). Different physical methods have been developed to enhance efficiency of naked pDNA delivery and improve distribution to target sites. They facilitate intracellular entry of pDNA into target cells by inducing transient penetrations in the cell membrane, making it permeable to pDNA.

Hydrodynamic delivery – Significant improvements of systemic pDNA injection can be achieved by a hydrodynamic delivery where the hydrodynamic pressure generated by rapid injection of a high volume of pDNA solution creates pores in cell plasma membranes (Liu and Knapp, 2001). This method is highly effective in small animals (Andrianaivo et al., 2004), but hardly applicable in clinical practice due to serious drawbacks connected to large injection volumes. However, computer-controlled injection devices for tissues-specific hydrodynamic delivery may have potential for use in gene therapy also in humans (Suda and Liu, 2015).

Gene gun – This delivery approach uses naked pDNA precipitated on golden microparticles that are propelled at high velocity into the target cells (Yang et al., 1990). Because poor penetration to deep organs limits the transfected area to superficial tissue layers like the skin, gene gun has found its main application in melanoma therapy (Wang et al., 2001) and DNA vaccination (Mahvi et al., 1997).

Electroporation – Electroporation-mediated delivery exposes cells to a short, high-intensity pulsed electric field that causes transient opening of small membrane pores permeable to naked pDNA. Efficient gene expression was reported in target sites, like skin (Maruyama et al., 2001), muscle (Hartikka et al., 2001), liver (Sakai et al., 2005), solid tumours (Matsuno et al., 2003), as well as in DNA vaccine applications (Frelin et al., 2010). However, such drawbacks as working range only in the proximity of electrodes, tissue damage caused by electric pulses, and invasiveness in non-topical applications limit the clinical use.

Sonoporation – Sonoporation utilizes ultrasound energy to perturb cell membranes by cavitation and microbubble formation, allowing direct entry of pDNA to the cytosol. Different tissues have been successfully transfected by using sonoporation alone (Danialou et al., 2002, Taniyama et al., 2002, Miller and Song, 2003) or in combination with liposome microbubbles (Suzuki et al., 2010). Sonoporation is both safe and non-invasive and has good potential for tissue- or organ-specific gene delivery.

Magnetofection – The principle of magnetofection is based on the delivery of pDNA associated with magnetic nanoparticles that are accumulated on the target cells by the application of external magnetic fields. Therefore, this method offers the possibility to remotely control targeting after systemic administration (Plank et al., 2003). Magnetofection has been effective in ex vivo transfection of endothelial cells (Gersting et al., 2004) and in vivo transfection in the gastrointestinal tract and blood vessels (Scherer et al., 2002).

2.2.2 Cationic lipid-based vectors

Cationic lipid-based vectors are typically monovalent or multivalent cationic lipids formulated as positively charged liposomes (Figure 2.2). All cationic lipids share common structure of a hydrophilic amine group that is connected via a linker structure to a hydrophobic tail. The amine head group is essential for binding with DNA, and the hydrophobic group composed of either fatty acid(s), alkyl, or cholesteryl moieties ensures assembling into bilayer vesicles (Brown et al., 2001). The cationic liposome formulations often contain neutral co-lipids such as dioleyl phosphatidylethanolamine (DOPE) (Felgner et al., 1994), dioleoyl phosphatidylcholine (DOPC) (Hui et al., 1996), dilauroyl phosphatidylcholine (DLPC) (Rodriguez-Pulido et al., 2009) and cholesterol (Liu et al., 1997) in order to improve stability of liposomes and to increase transfection efficacy.

Cationic liposomes are self-assembly systems that spontaneously complex and condense DNA via electrostatic interactions between anionic DNA phosphates and the positively charged amine head group of the lipid. The resulting cationic liposome/DNA complexes (lipoplexes) exist in a multilamellar (L_α^C) structure with alternating lipid bilayers and DNA monolayers or in an inverted hexagonal (H_{II}^C) phase where DNA is encapsulated within inverse micellar tubules (Safinya, 2001). The lipoplexes with a net positive charge readily associate with the negatively charged cell surfaces and have been shown to mediate the highest levels of transfection (Sakurai et al., 2000). Lipoplexes enter cells primarily via endocytosis (Simões et al., 1999) and escape from endosomes due to interactions with anionic phospholipids in the endosomal membrane. Cationic lipids destabilize the endosomal membrane due to the formation of cationic–anionic ion pairs, which causes reorganization of anionic phospholipids and forming the hexagonal phase, followed by dissociation and release of nucleic acid from the lipoplex (Xu and Szoka Jr, 1996).

Since the first cationic lipid DOTMA was introduced as a gene carrier (Felgner et al., 1987), various lipoplex formulations have been effective in DNA delivery to cultured cells and some have become commercially available as in vitro transfection reagents, for example combinations of DOTMA/DOPE as Lipofectin®, DOSPA/DOPE as Lipofectamine®, and DOGS as Transfectam®.

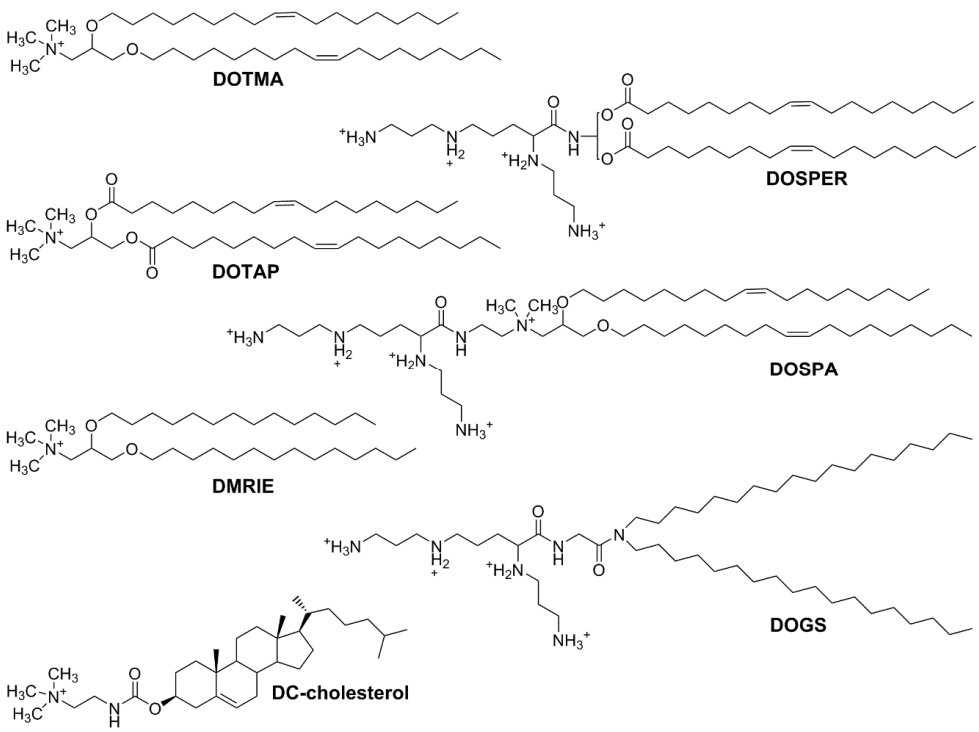


Figure 2.2 *Cationic lipids commonly used for gene delivery.*

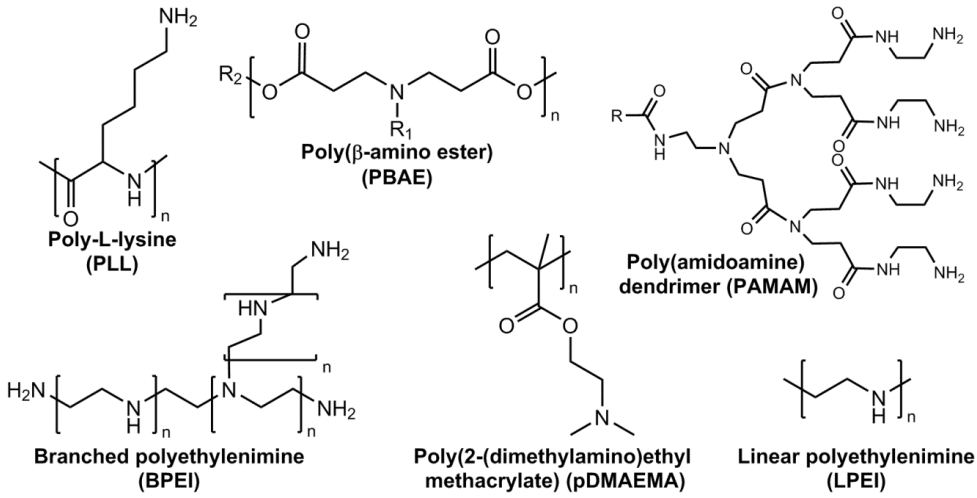


Figure 2.3 *Cationic polymers commonly used for gene delivery.*

2.2.3 Cationic polymer-based vectors

Various cationic polymers such as poly(L-lysines), poly(ethylenimines), poly(beta-amino esters), poly(dimethylaminoethyl methacrylates), dendrimers and carbohydrate-based polymers (e.g. chitosan, cyclodextrin and dextran) have been used to bind and condense nucleic acids into gene delivery complexes - polyplexes. They contain different amine functional groups ($R–NH_2$, $R_2–NH$, $R_3–N$), which can be protonated in the physiological pH range (5.0–7.4) and bind with negatively charged phosphate groups on nucleic acids. The structures of the most widely studied cationic polymers are shown in Figure 2.3.

Cationic polymer-based vectors constitute an alternative to cationic lipids as a result of their versatile structure, which can be easily modified to favour biodegradability or to serve as a scaffold to covalently attach targeting ligands. The large design flexibility of cationic polymers enables enhanced vector development by using high-throughput combinatorial approach for synthesis and screening of polymer libraries (Anderson et al., 2003). Compared with lipoplexes, polyplexes condense DNA more efficiently and offer better protection against enzymatic nucleic acid degradation (Ruponen et al., 1999), have an advantage of higher stability, and allow greater manipulation of their physical characteristics (Goodwin and Huang, 2014).

Polylysines

Poly-L-lysine (PLL) belongs to the first generation of polymeric transfection vectors. Its use was demonstrated already in 1987 as asialooromucoid-PLL conjugate for targeted gene transfer to hepatocytic cells (Wu and Wu, 1987). PLL is a linear polymer available in a wide range of molecular weights (4–200 kDa). PLL has high affinity for DNA binding (Laemmli, 1975) but only PLLs with molecular weights exceeding 3 kDa can effectively condense DNA to stable complexes, suggesting the importance of primary amine number for complex formation (Kwoh et al., 1999). PLL contains only primary ε-amines on the side chains, with a pK_a value around 10.8. Based on the high pK_a, all amino groups in PLL are protonated at physiological pH, thus having no extra buffering capacity for assisting endosomal escape (Tang and Szoka, 1997). Therefore, PLL polyplexes are unable to avoid trafficking to lysosomes, which results in low transfection efficiency (Akinc and Langer, 2002). To prevent accumulation in lysosomal compartments, the addition of lysosomotropic chloroquine (Cotten et al., 1990), membrane-active peptides (Wagner et al., 1992a), or substituting PLL with tryptofan (Wadhwa et al., 1997) and histidine groups (Midoux and Monsigny, 1999) have been required to increase transfection efficiency. A variety of different targeting ligands, such as lactose (Midoux et al., 1993), galactose (Hashida et al., 1998), asialoglycoprotein (Chowdhury et al., 1993), antibodies (Suh et al., 2001), folate (Mislick et al., 1995), and transferrin (Wagner et al., 1992b), have been conjugated to PLL in order to improve cell surface binding and internalization. PLL/DNA complexes have displayed a relatively high toxicity that significantly increases with molecular weight (Choi et al., 1998). To improve systemic tolerability and increase the stability of PLL/DNA complexes in the presence of serum proteins, different block

(Katayose and Kataoka, 1997) and graft (Toncheva et al., 1998) copolymers of PEG–PLL have been used to mask the positive charge of polycations.

Polyethylenimines

Polyethylenimine (PEI) is one of the most potent cationic polymeric vectors available because of its simplicity, stability, and relatively high transfection efficiency in vitro and in vivo. PEI is well-known not only as a model transfection agent but also as a promising candidate in several clinical trials (Table 2.2).

PEI molecule, a simple repetition of the 43 Da ethyleneimine units, exists in either linear or branched form. Branched PEI (BPEI) is water-soluble and has an extremely high cationic charge density due to the fact that every third atom of the PEI being a potentially protonable amino nitrogen. BPEI has positive charges on a randomly branched backbone where tertiary amines represent branching points. The theoretical ratio of primary to secondary to tertiary amines is 1:2:1. However, by using NMR analysis Von Harpe et al. (2000) estimated that commercially available compounds have a ratio close to 1:1:1, which indicates an even higher degree of branching. The average pK_a of BPEIs lies between 7.4 and 8.5 (Von Harpe et al., 2000, Choosakoonkriang et al., 2003). The unprotonated amines allow an effective buffering capacity over a broad pH range (Tang and Szoka, 1997). Only ~19% of BPEI amino nitrogens are protonated at physiological pH and ~25% can be protonated during acidification in endosomes (Suh et al., 1994), thus facilitating endosomal release by the proton sponge effect. In contrast to BPEI, linear PEI (LPEI) contains only secondary amines. The average pK_a values for LPEI are similar to those for BPEI (Choosakoonkriang et al., 2003, Ziebarth and Wang, 2010).

PEIs are available in a variety of molecular weights, ranging from 0.7 to 800 kDa. However, the optimum molecular weight for DNA gene delivery lies between 5 and 25 kDa (Neu et al., 2005), as the molecular weight has been shown to strongly correlate not only with transfection efficacy (Godbey et al., 1999a) but also with cytotoxicity (Fischer et al., 2003). PEI is a non-degradable polymer and exhibits significant dose-dependent toxicity. It has been proposed that PEI-induced cytotoxicity involves early necrotic-like membrane damage and late mitochondrial-mediated apoptotic cell death (Moghimi et al., 2005) and originates from free PEI polymers rather than from PEI polyplexes (Boeckle et al., 2004). Branched 25-kDa PEI was also found to alter expression levels of genes related to oxidative stress, inflammatory signalling, and cytotoxicity (Beyerle et al., 2010).

Transfection efficacy and toxicity of PEI are also strongly dependent on the polymer topology (Breunig et al., 2007a). LPEI has been described in numerous studies as a much more efficient delivery vector than its branched form (Goula et al., 1998a, Kircheis et al., 1999, Bragonzi et al., 2000, Wightman et al., 2001, Verbaan et al., 2004). This finding may derive from LPEI polyplexes being less stable than BPEI polyplexes (Wightman et al., 2001). The lower stability of LPEI polyplexes may help during intracellular release (Itaka et al., 2004) and nuclear entry (Brunner et al., 2002). Details of the mechanisms of PEI-mediated DNA delivery are presented in Section 2.3.

Although PEI structure was simply chosen from a chemical catalogue as an organic macromolecule with the highest cationic-charge-density potential (Boussif et al., 1995), various PEI formulations have become effective for in vitro delivery of pDNA (Boletta et al., 1997, Ferrari et al., 1997), ODNs (Merdan et al., 2002, Brus et al., 2004), ribozymes (Aigner et al., 2002), and siRNA (Zintchenko et al., 2008). Crosslinking of low-molecular-weight LPEIs with various biodegradable bonds (Breunig et al., 2007a), grafting PEG (Petersen et al., 2002a), and shielding of polyplexes with hyaluronic acid (Hornof et al., 2008) have been used to improve biocompatibility and reduce cytotoxicity.

PEIs have been further modified with various receptor ligands to add target specificity for in vivo applications. For example, transferrin (Kircheis et al., 2001a), folate (Guo and Lee, 1999), and epidermal growth factor (Blessing et al., 2001) were used for targeting tumour cells, galactose (Morimoto et al., 2003) for liver cells, mannose (Diebold et al., 1999) for dendritic cells, and RGD peptide (Kunath et al., 2003) for integrin receptors on endothelial cells. Also different antibodies coupled to PEI have been used as efficient targeting ligands: anti-CD3 antibody to achieve delivery to peripheral blood mononuclear cells (O'Neill et al., 2001), antigen binding fragment (Fab′) of the OV-TL16 antibody to target human ovarian carcinoma cells (Merdan et al., 2003), anti-HER2 antibody (trastuzumab) to target breast cancer cells (Chiu et al., 2004), and anti-GAD (glutamic acid decarboxylase) antibody to target islet beta cells (Jeong et al., 2005).

Poly(beta-amino esters)

Poly(beta-amino esters) (PBAEs) are biodegradable cationic polymers that are readily synthetized by the conjugate addition of amines to diacrylates (Lynn and Langer, 2000). The one-step reaction allows use of high-throughput synthesis and screening techniques to create a vast library of structurally unique PBAE structures (Anderson et al., 2003).

PBAEs have great potential to overcome the cytotoxicity problems seen with the first generation of non-viral vectors. Unlike PLL and PEI, PBAE polymers are non-cytotoxic and biodegradable. They degrade via hydrolytically cleavage of ester groups to non-toxic byproducts (1,4-butanediol and β-amino acids) with a degradation half-life of hours to days (Lynn et al., 2001). The lead polymer structures are linear ~10 kDa PBAEs synthetized at an amine/acrylate ratio of 1.2:1 with hydroxyl side chains and primary amine end groups (Green et al., 2008). They efficiently bind and condense DNA to form cationic polyplexes of 50–200 nm, have a high cellular uptake, and can facilitate endosomal escape by similar mechanisms as PEI (Akinc et al., 2003).

In contrast to 25-kDa BPEI, PBAEs have a 4–8-fold higher transfection efficiency and considerably lower toxicity (Green et al., 2006). End-modified PBAEs that contained terminal primary amines were found to have comparable in vitro transfection efficacy to adenoviral vector. The high transfection efficiency was mainly attributed to the improved DNA condensation and increased cellular uptake (Green et al., 2007). In vivo, PBAEs delivered DNA 4-fold better than jetPEI (commercial

transfection reagents based on 22-kDa LPEI) and 26-fold better than naked pDNA and effectively inhibited tumour growth (Anderson et al., 2004).

2.2.4 Hybrid polymer-based delivery systems

Combining different vectors into a single gene delivery system has been successfully applied to improve gene transfer and modulate cytotoxicity of the first generation of non-viral vectors. One such strategy utilizes delivery systems based on noncovalently modified polyplexes. Noncovalent modifications focus on either (i) the encapsulation of polyplexes into PEG-stabilized liposomes (Ko et al., 2009) or (ii) the formation of ternary complexes of cationic liposome, cationic polymer, and nucleic acid – lipopolyplexes.

Lipopolyplexes (LPPs) are formed by mixing polyplexes with preformed cationic liposomes that spontaneously rearrange to form particles with a condensed DNA core and an outer lipidic shell (Gao and Huang, 1996). They combine favourable delivery properties of their parent delivery vectors: the outer cationic liposome shell determines increased cellular uptake and low toxicity, whereas the cationic polymer ensures effective nucleic acid condensation and facilitates endosomal release. The individual vector components may also act simultaneously, enhancing gene transfer (Lampela et al., 2003, Lee et al., 2003).

Various LPP formulations containing PLL, PEI, or PBAE polyplexes have been described as promising reagents for both in vitro and in vivo DNA delivery (Table 2.1). The enhanced in vitro transfection efficiency was mainly attributed to decreased cytotoxicity and improved colloidal stability in the presence of serum proteins and at physiological salt concentrations. For example, LPP formulations of 25-kDa BPEI in combination with DOTAP/cholesterol liposomes mediated higher levels of transgene expression together with lower cytotoxicity (Lee et al., 2003, Penacho et al., 2010) and better serum resistance (Garcia et al., 2007, Urbiola et al., 2013) compared with polyplex and lipoplex alone. To improve systemic gene delivery, various PEG-lipid conjugates together with (Hu et al., 2010) or without (Nie et al., 2011) targeting ligands were incorporated into LPPs. Besides DNA delivery, transferrin-conjugated pH-sensitive LPPs were shown to be effective for antisense ODN delivery in acute myeloid leukaemia therapy (Jin et al., 2010) or PEGylated NGR (aspargine-glycine-arginine) peptide targeted lipoplexes for efficient delivery of siRNAs into solid tumours in mice (Chen et al., 2010).

Another strategy to enhance delivery capacity of synthetic carriers involves hybrid vectors that incorporate both viral and non-viral components. As examples, adenoviral protein-PLL-transferrin-DNA complexes use the capacity of adenoviruses to disrupt endosomes for enhanced endosomal escape (Curiel et al., 1991) and retrovirus-like particle PEI complexes combine mechanisms of PEI-mediated cell entry and retroviral intracellular processing (Ramsey et al., 2010).

Table 2.1 *Examples of lipopolyplexes used for DNA delivery.*

Components of lipopolex		Important lipopolyplex (LPP) characteristics and delivery strategy	References
Liposome	**Polyplex**		
DOTMA	BPEI 25 kDa	LPP displaying high gene expression in lungs, liver, and spleen after intravenous injection to mice	(Matsumoto et al., 2008)
DOTAP	PBAE 10 kDa	LPP based on biodegradable PBAE transfecting primary human aortic endothelial cells and smooth muscle cells with high efficiency, with possible application in gene-eluting stent delivery system for treatment of coronary restenosis	(Brito et al., 2008, and 2010)
DOTAP/Chol	BPEI 2 kDa	Transferrin-associated LPP	(Penacho et al., 2010)
DOTAP/Chol	LPEI 22 kDa	Targeted LPP containing folic acid capable of transfecting therapeutic IL-2 gene in cancer cell lines at very high serum concentrations (60% FBS)	(Urbiola et al., 2013)
DOSPER	BPEI 25 kDa	Transcriptionally targeted LPP transfecting IL-2 gene to colon cancer cells in both monolayer and multicellular spheroid cultures with 1300-fold higher gene expression than the corresponding lipoplex	(Gaedtke et al., 2007)
DOCSPER	sPLL 2.6 kDa	LPP based on short PLL with 18 L-lysine residues with enhanced gene transfer to primary porcine smooth muscle cell cultures (5-fold) and in vivo local delivery to porcine femoral arteries (1.5-fold) compared with lipoplex	(Golda et al., 2007)
Histidylated Chol	BPEI 25 kDa	LPP-mediated gene delivery into mesenchymal stem cells derived from bone marrow under serum conditions (10% FBS) with higher efficiency than the best performing polyplex	(Song et al., 2012)
DOPE/Chol-PEG	LPEI 22 kDa	PEGylated LPP with pH-cleavable pyridylhydrazone-based Chol-PEG for shielding in extracellular compartments and dynamic deshielding in endosomes; contains B6 targeting peptide conjugated on the LPP surface to target TfR in prostate cancer cells	(Nie et al., 2011)
POPC DSPE-PEG2k-biotin	BPEI 25 kDa	PEGylated LPP formulated by combining polyplexes and anionic liposomes with conjugated SA/MAb for transfection of liver cancer cells in vitro and in vivo	(Hu et al., 2010)
EPC/EPG/Chol	pDMAEMA	Targeted LPP prepared by coating polyplex with anionic lipids and coupling Fab' fragments directed against epithelial glycoprotein-2 on LPP's surface	(Mastrobattista et al., 2001)
DPPC/Chol DPPC/DPPG	BPEI 4-10 kDa	LPP based on rigid, negatively charged lipids showed enhanced biological activity and absence of aggregation compared with corresponding polyplex	(Schäfer et al., 2010)

Chol: cholesterol; IL-2: interleukin 2; TfR: transferrin receptor; POPC: 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine; DSPE: distearoylphosphatidylethanolamine; SA/MAb: streptavidin/monoclonal antibody; EPC: egg phosphatidyl-choline; EPG: egg phosphatidylglycerol; pDMAEMA: poly(2-(dimethylethylamino)ethyl methacrylate; Fab': fragment antigen-binding; DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DPPG: 1,2-Dipalmitoyl-sn-glycero-3-phosphorylglycerol

2.3 Mechanisms of PEI-mediated DNA delivery

2.3.1 Structure of PEI polyplexes

PEI polyplexes are spontaneously formed as a result of self-assembly systems based on electrostatic interactions between the positive charges of PEI amines and the negative charges of the phosphate backbone of the DNA. For optimal transfection, polyplexes are usually formed with an excess of PEI (i.e. N/P ratios of 6–12), where about 86% of PEI was found to be free, not bound to the DNA (Clamme et al., 2003).

The binding process of PEI to DNA has been shown to proceed in two modes: (i) simple binding to DNA groove and (ii) binding to external phosphate backbone that involved DNA condensation (Utsuno and Uludag, 2010). The condensed DNA was observed to remain in the B-form, indicating that the formation of PEI polyplexes does not interfere with the secondary structure of DNA (Choosakoonkriang et al., 2003).

PEI–DNA polyplexes form mainly toroidal structures with multiple copies of pDNA in a single toroid (Tang and Szoka, 1998). Toroidal particles appeared to be in a size range of 40–60 nm in diameter under an electron microscope, but may cluster into larger aggregates in solution (Tang and Szoka, 1997). The size of polyplexes differs depending on PEI topology and the ionic strength of the media used during complexation. In low-salt buffers, both BPEI and LPEI polyplexes are small (<100 nm) and retain their size over a prolonged incubation time due to ionic repulsion. However, after addition of salt to physiological levels, the presence of extra ions masks surface charges and causes aggregation. The speed of aggregation is affected by PEI branching. In contrast to LPEI polyplexes, which are known to rapidly form large aggregates, BPEI polyplexes aggregate very slowly (Goula et al., 1998b, Wightman et al., 2001).

2.3.2 Cell surface binding and intracellular uptake

The binding of non-targeted PEI polyplexes to cell surface is mediated by nonspecific, electrostatic interactions between positively charged polyplexes and highly negatively charged cell-surface proteoglycans (Mislick and Baldeschwieler, 1996). Cell-surface proteoglycans are ubiquitously expressed on the plasma membrane and consist of a core protein with one or more covalently attached glycosaminoglycan chains (Figure 2.4). Glycosaminoglycans (GAGs), such as heparan sulfate (HS) and chondroitin sulfate (CS), comprise repeating disaccharide building blocks of amino sugar (HS: *N*-acetyl-glucosamine, CS: *N*-acetylgalactosamine) and uronic acid (HS and CS: glucuronic acid, HS: iduronic acid) (Esko et al., 2009).

It has been shown that cell-surface GAGs influence several aspects of polyplex-mediated gene transfer. GAGs can have a protective role against polycation-associated cytotoxicity (Belting and Petersson, 1999). However, they can readily disrupt polyplex integrity in the extracellular space (Ruponen et al., 1999) and significantly alter or inhibit gene transfection (Ruponen et al., 2004). Unlike polyplexes without any ligand,

targeted polyplexes bind to cell surfaces specifically via ligand-receptor interactions. It has been reported that PEI polyplexes enter cells through diverse endocytic pathways such as clathrin-mediated endocytosis, caveolae-mediated endocytosis, micropinocytosis, and clathrin- and caveolin-independent endocytosis (Midoux et al., 2008). It is also noteworthy that several uptake mechanisms can occur simultaneously in the same cell. The explanation for the variety of mechanisms can be attributed to differences in polyplex formulations, cell types, and experimental design of uptake studies. The study by von Gersdorff et al. (2006) showed that the internalization route mediating efficient gene expression is both cell type- and polyplex type- dependent. Smaller polyplexes (~100 nm) were taken up by clathrin-dependent and caveolae-dependent routes, whereas larger polyplexes (>1 μm) entered cells by clathrin- and caveolin-independent endocytic pathways. In contrast to clathrin-mediated endocytosis, the caveolar uptake of polyplexes leads to degradation in lysosomes (Rejman et al., 2005) and plays an important role in successful gene transfection (Van Der Aa et al., 2007, Gabrielson and Pack, 2009).

Compelling evidence indicates that syndecans, which are transmembrane heparan sulfate proteoglycans, act as endocytic receptors for polyplexes and mediate the internalization via binding to actin filaments and engulfment of the plasma membrane (Kopatz et al., 2004). Männistö et al. (2007) suggested that the amounts of cell-surface GAGs changes during the cell cycle and can influence the cellular uptake of polyplexes. Cell-surface GAGs have been found to channel polyplexes mainly to clathrin- and caveolae-independent endocytic pathways (Payne et al., 2007). Furthermore, Paris et al. (2008) demonstrated that the type of cell-surface proteoglycans involved in polyplex binding determines the cell uptake rate. In their study, binding to syndecan-1 (has both HS and CS) was found to facilitate rapid internalization, whereas binding to syndecan-2 (has only CS) delayed cellular entry and inhibited transfection.

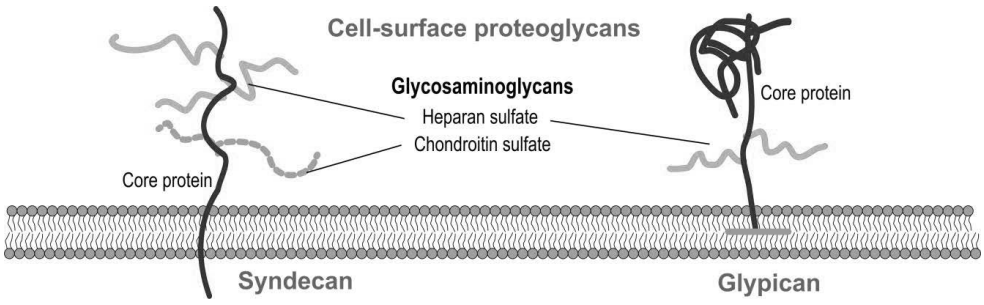


Figure 2.4 *Schematic representation of cell-surface proteoglycans.*

2.3.3 Endosomal escape

Once internalized in the cell, polyplexes must escape from the endosomal pathway before the rapid acidification of endosomes and eventual enzymatic degradation in lysosomes. Because of many unprotonated amines, PEI displays a strong buffering capacity over a broad pH range (Suh et al., 1994). It has been proposed that this special property enables endosomal release to be triggered thought a so-called "proton-sponge effect" (Boussif et al., 1995).

The hypothesis postulates that unprotonated amines of PEI are capable of absorbing protons that are pumped inside the endosome by proton pump vacuolar ATPase during endosome maturation (Figure 2.5). This "proton-sponge" activity perturbs rapid endosomal acidification, leading to a greater influx of protons, followed by an enhanced passive influx of chloride ions and water. The osmotic swelling together with an increase in volume caused by polymer swelling causes rupture of the endosomal membrane and subsequent release of polyplexes into the cytosol, prior to the fusion with lysosomes (Behr, 1997).

Although the proton-sponge hypothesis is a widely supported release mechanism (Thomas and Klibanov, 2002, Sonawane et al., 2003, Akinc et al., 2005), some evidence suggests that it cannot be the sole mechanism of endosomal release (Funhoff et al., 2004, Won et al., 2009, Benjaminsen et al., 2013). It has been proposed that polyplexes accumulate in lysosomes, where they can induce small, local rupturing of the lysosomal membrane by either osmotic rupture or direct binding of PEI aggregates to the inner membrane surface (Bieber et al., 2002). A study by Rehman et al. (2013) provided supporting evidence for a proton-sponge effect: using live cell imaging, their findings suggested that the protonation of PEI polyplexes causes a close interaction with the inner surface of endosomal membrane, where a very local osmotic or mechanical effect leads to transient membrane destabilization rather than to complete rupture.

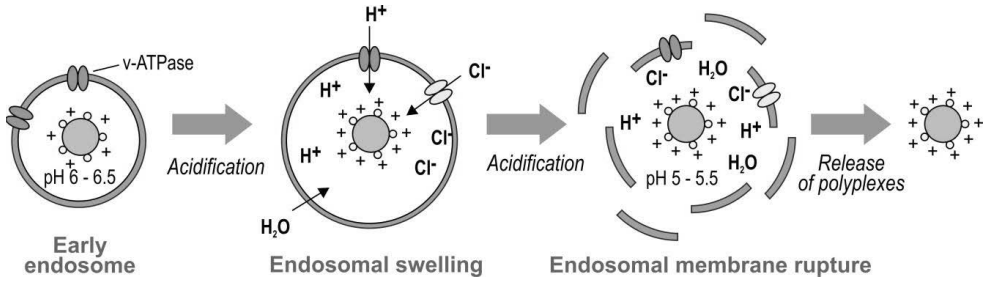


Figure 2.5 *Schematic representation of the proton-sponge mechanism.*

2.3.4 Cytoplasmic transport and DNA release

Once polyplexes enter the cytosol, they must diffuse through the dense network of the cytoskeleton to reach the nucleus and at the same time resist degradation by cytosolic nucleases. It remains, however, unclear whether polyplexes go through cytoplasmic trafficking before or after their endosomal release (Beckert et al., 2015). Several studies have pointed out that polyplexes still inside endosomal vesicles are actively transported towards the nucleus along microtubules by molecular motors such as dynein or kinesin (Suh et al., 2003, Bausinger et al., 2006, Grosse et al., 2007). Once inside the cytoplasm, polyplexes are unable to efficiently utilize microtubule-mediated transport and their migration through the cytoplasm by passive diffusion is very slow (Nguyen and Szoka, 2012). Therefore, the optimal place for endosomal release is in a perinuclear region (Lechardeur et al., 2005), where polyplexes have been proposed to have the best chance to enter the nucleus (Dinh et al., 2007).

It is generally believed that DNA should dissociate from the polyplex before transcription. However, it is not completely clear whether polyplexes disassemble before or after nuclear entry. DNA dissociation from the PEI polyplex may occur via competitive binding and ion exchange with cytosolic polyanions, RNA, nuclear natural polyamines, or chromatin (Schaffer et al., 2000, Huth et al., 2006). Also, it is highly probable that released DNA might not remain completely free and immediately associate with other polycations present in the cytosol (Dean et al., 2005). Some studies have indicated that PEI polyplexes dissociate in the cytosol and release DNA prior to nuclear entry (Okuda et al., 2004, Won et al., 2009). These finding are, however, in contradiction to numerous reports on the presence of intact polyplexes in the nucleus (Pollard et al., 1998, Godbey et al., 1999b, Grosse et al., 2004, Itaka et al., 2004, Mishra et al., 2004, Männistö et al., 2005, Breunig et al., 2007b, Breuzard et al., 2008, Chen et al., 2008, Cohen et al., 2009), suggesting that PEI may remained partly complexed with DNA in the nucleus.

2.3.5 Nuclear entry

Free or complexed DNA must penetrate the nuclear envelope in order to be transcribed and the resulting mRNA translated. However, plasmid DNA nuclear import has been shown to be a rate-limiting step in PEI-mediated delivery. Cohen et al. (2009) estimated by using quantitative PCR that only 1–5% of the initial DNA dose is delivered to the nucleus by PEI polyplexes.

In actively dividing cells, BPEI polyplexes have been reported to enter the nucleus by passive nuclear uptake during mitosis (Brunner et al., 2000, Grosse et al., 2006). By contrast, the nuclear uptake of LPEI polyplexes was cell cycle-independent and proceeded via unknown mechanisms (Brunner et al., 2000). In non-dividing cells, the only possible route for nuclear entry is active transport through the nuclear pore complex (NPC). The inner diameter of the NPC (~9 nm) allows passive diffusion only for smaller macromolecules (≤ 40 kDa) or DNA molecules of 210–350 bp. Therefore, active transport of either free or complexed DNA via NPC has to be mediated by

nuclear localization signal (NLS) sequences that initiate nuclear import (van der Aa et al., 2006). Various studies have focused on mimicking this route by coupling NLS sequences with PEI polyplexes. Although linking of NLS either to PEI (Ogris et al., 2001) or directly to the DNA backbone (Brandén et al., 1999, Zanta et al., 1999) enhanced transfection efficiency, some studies showed contradictory findings where NLS peptide failed to facilitate DNA nuclear translocation (van der Aa et al., 2005).

As many studies have found that polyplexes exist intact in the nucleus, the presence of PEI may have an influence also on transcriptional activity of DNA. According to early studies using acellular transcription (Bieber et al., 2002) and nuclear microinjection (Honore et al., 2005), the complete unpacking of polyplexes was not necessary for transcription, but the accessibility of DNA to transcription machinery was influenced by the extent of the DNA condensation. Only polyplexes at N/P ratios 5–15 were transcribed as efficiently as free DNA. Conversely, it has been observed that transcriptional/translational activity of DNA was strongly reduced by a close interaction with PEI polyplexes (Breunig et al., 2007b, Cohen et al., 2009, Glover et al., 2010).

2.4 Non-viral gene delivery vectors in clinical trials

In just over 25 years, more than 2400 gene therapy clinical trials throughout the world have evaluated various gene-based drug candidates, mainly as a therapy for cancer diseases (64.6%), monogenic diseases (10.5%), cardiovascular diseases (7.4%), infectious diseases (7.4%), neurological diseases (1.8%) and ocular diseases (1.4%) (as of April 2017, The Journal of Gene Medicine, Gene Therapy Clinical Trials worldwide database, at http://www.wiley.co.uk/genmed/clinical).

However, only one-fourth of these clinical trials employ non-viral vectors. In fact, mostly naked pDNA (17.1%) and cationic lipid–DNA complexes (4.6%) have been used for gene delivery, whereas the role of promising polymeric vectors is still in the preclinical stages of development. Table 2.2 gives an overview of approved, on-going, and completed clinical studies with non-viral carriers during the last two decades. A significant number of gene therapy clinical trials are still in phase I and phase I/II (95%), and therefore, bringing a safe and efficient non-viral gene therapy product to market will take time (Ginn et al., 2013).

Table 2.2 *Non-viral gene delivery systems tested worldwide in clinical trials (as of July 2015).*

Delivery system	Gene therapy drug / gene construct	Administration	Target disease	Phase	Reference/ Trial registration[1]
Carrier-free					
Naked pDNA	pDNA coding human VEGF	Intramyocardial	Angina pectoris	II/III	(Stewart et al., 2009)
	Encapsulated CYP2B1-expressing cells	Intratumoural	Pancreatic carcinoma	I/II	(Lohr et al., 2001)
	Vaccine - MUC1 transfected dendritic cells	Subcutaneous	Breast carcinoma	I/II	(Pecher et al., 2002)
	pDNA encoding HGF	Intramuscular	Critical limb ischaemia	II	(Powell et al., 2010)
	pDNA encoding human dystrophin	Intramuscular	Muscular dystrophy	I	(Romero, 2004)
Naked mRNA	CV9130 vaccine - mRNA encoding tumour antigens	Intradermal	Prostate cancer	I/IIa	(Kubler et al., 2015)
Gene gun	Vaccine - autologous cells transfected with IL-12	Subcutaneous	Malignant melanoma	I	(Sun et al., 1998)
Electroporation	pDNA encoding IL-2	Intratumoural	Malignant melanoma	II	NCT01174238
	VGX-3100, pDNA encoding E6 and E7 proteins of HPV	Intramuscular	Cervical neoplasia	IIb	NCT01304524
	mRNA, autologous c-Met redirected T cells	Intratumoural	Breast cancer	I	NCT01837602
Lipid-based					
DOSPA/DOPE	Vaccine - IL-2 transfected secreting fibroblasts	Intradermal	Malignant tumour	I	(Veelken et al., 1997)
GL-67/DOPE/DMPE-PEG	pDNA encoding CFTR	Inhalation	Cystic fibrosis	I/II	NCT00789867
TMAG/DLPC/DOPE	pDNA encoding IFN-β	Intratumoural	Malignant glioma	I	(Yoshida et al., 2004)
DMRIE/DOPE	Leuvectine®, pDNA encoding IL-2	Intraprostatical	Prostate cancer	I/II	NCT00005072
	Allovectin-7®, pDNA encoding HLA-B7/β2 microglobulin	Intralesional	Metastatic melanoma	III	NCT00395070
GAP-DMORIE/DPyPE	Tetravalent dengue pDNA vaccine	Intramuscular	Dengue disease	I	NCT01502358

Delivery system	Gene therapy drug / gene construct	Administration	Target disease	Phase	Reference/ Trial registration[1]
DOTAP/Chol	pDNA expressing BikDD gene	Intravenous	Pancreatic cancer	I	NCT00968604
	TUSC2 expression pDNA	Intravenous	Lung cancer	I	NCT00059605
DOTMA/DOPE	pDNA encoding VEGF	Intracoronal	Coronary artery stenosis	I/II	(Laitinen et al., 2000)
Polymer-based					
linear PEI	CYL-02, pDNA encoding sst2+dck:umk genes	Intratumoural	Pancreatic carcinoma	I	NCT01274455
linear PEI	BC-819, pDNA encoding DTA	Intravesical	Bladder cancer	II	NCT00595088
linear PEI	ex vivo transfection of circulating mononuclear cells with human eNOS-pVAX plasmid	Intracoronal	Acute myocardial infarction	IIb	NCT00936819
linear PEI	SNS01-T, RNAi-resistant pDNA encoding eIF5a and siRNA	Intravenous	Multiple myeloma, B cell lymphoma	I/II	NCT01435720
linear PEI-mannose	DermaVir, vaccine - pDNA encoding HIV antigens	Topical (patches)	HIV/AIDS infection	II	NCT00711230
PEG-PEI-Cholesterol	EGEN-001, IL-12 expressing pDNA	Intraperitoneal	Ovarian, fallopian tube and peritoneal cancer	II	NCT01118052
PEG-CK30	pDNA encoding CFTR	Intranasal	Cystic fibrosis	I	(Konstan et al., 2004)
CDP-PEG-Tf	siRNA against RRM2	Intravenous	Solid tumours	I	NCT00689065
Poloxamer (CRL1005) + benzalkonium chloride	ASP0113 vaccine - pDNA encoding gB, pp65, hCMV immunogens	Intramuscular	CMV infection	III	NCT01877655

[1]ClinicalTrials.gov identifier, https://clinicaltrials.gov/show/NCT Number; VEGF: vascular endothelial growth factor; CYP2B1: cytochrome P450 form; MUC1: human tumour antigen mucin; c-Met: cell surface receptor for HGF; HGF: hepatocyte growth factor; IL: interleukin; HPV: human papilloma virus; GL-67: lipid #67 3-β-(sperminecarbamoyl) cholesterol; DMPE: dimyristoylphosphatidylethanolamine; CFTR: cystic fibrosis transmembrane regulator gene; TMAG: N-(a-trimethyl ammonioacetyl)-didodecyl-D-glutamate chloride; DLPC: dilauroyl phosphatidylcholine; IFN-β: interferon β; HLA: human leukocyte antigen; GAP-DMORIE: (±)-*N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-bis(*cis*-9-tetradecenyloxy)-1-propanaminium bromide; DPyPE: 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamin; BikDD: mutant of proapoptotic Bcl-2 interacting killer; *TUSC2*: tumor suppressor gene; sst2: somatostatin receptor subtype 2; dck::umk: deoxycitidine kinase:uridylmonophosphate kinase; DTA: diphteria toxin-A; eNOS: endothelial nitric oxide synthase; RRM2: ribonucleotide reductase subunit 2; CK30: polycationic 30-mer lysine peptide; CDP: cyclodextrin-containing polymer; Tf: human transferrin protein; gB:glycoprotein B; pp65: tegument phosphoprotein; hCMV: human cytomegalovirus.

3 Aims of the study

The primary objective of this thesis was to study polymer–based gene delivery vectors with respect to the mechanisms underlying DNA complex formation and intracellular gene delivery processes.

Specific aims were the following:

1. To investigate the mechanisms of polyplex formation by time-resolved fluorescence spectroscopy approach and compare the polymer–DNA interaction and binding of cationic polymers with various structures and transfection efficiencies.

2. To evaluate the effects of polymer–DNA binding affinity on transfection efficiency and to determine whether any clear and robust relationship exists between these parameters. Such a relationship would predict possible behaviours of polyplexes in transfection and provide guidelines for the selection of optimal polymeric structure.

3. To examine the role of free polymer in polyethylenimine-mediated gene delivery and to clarify how the interactions between free polymer and abundant glycosaminoglycans on the cell surface influence gene delivery efficacy.

4. To assess the role of polyethylenimine structure in a lipopolyplex–mediated gene transfection and to elucidate the polymer properties responsible for the synergistic enhancement of transfection efficiency in vitro.

4 Overview of the methods

4.1 Polymer–DNA complex preparation

Detailed descriptions of materials and methods can be found in the original publications (**I–VI**). All DNA complexes used in this study were self-assembly systems, which formed spontaneously as a result of polyion coupling reaction after vigorous mixing of pDNA and polycation solution. The ionic balance of polyplexes was determined as an N/P ratio, i.e. the molar ratio of protonable amines on polycation to the negatively charged phosphates in the DNA. The properties of cationic carriers and plasmids are listed in Table 4.1 and Table 4.2, respectively. PEIs, PLL and DOSPER were commercially available and used without further purification. PBAEs were synthesized and characterized in the David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA, USA. Polyplexes were prepared by either a direct or a stepwise method and incubated for the necessary time to reach equilibrium before further characterization.

4.1.1 Direct method

In the direct method (**I, II, V, VI**), the final N/P ratio of the resulting polyplex was reached with the single addition of polymer solution to DNA solution at a volume ratio of 1:1. Such polyplexes formed with BPEI are denoted as “nonpurified” in Studies **I** and **V**. Nonpurified polyplexes with initial an N/P ratio of 6 were further purified by the use of size exclusion chromatography or electrophoresis resulting in “purified” polyplexes that had an N/P ratio close to 3. “Spiked” polyplexes referred to the purified polyplexes where extra BPEI solution was added to restore the original N/P ratio of 6 (Figure 4.1).

LPPs studied in Study **VI** were produced by mixing BPEI or LPEI polyplexes (direct method) with preformed DOSPER liposomes at various ratios. For LPPs, two key parameters were used: (i) an N/P ratio during polyplex formation and (ii) a lipid/DNA ratio during LPP formation. The tested DOSPER/DNA mass (w/w) ratios of 2.5, 5, 7.5, and 10 corresponded to the molar N/P ratios of 1.2, 3, 6, 9 and 12, respectively.

4.1.2 Stepwise method

The stepwise method, which is independent of the final N/P ratio, was used in fluorescence measurement studies (**I, III, IV**). An initial polyplex solution with N/P ratio of 0.2 (**I, III**), 0.4 (**I**) and 0.6 (**IV**) was prepared by mixing equal volumes of DNA and polymer solution. The complexation was followed by the measurement of fluorescence spectrum and then the next N/P ratio was adjusted by the addition of the

appropriate amount of polymer solution. The measured N/P ratios for each polymer are shown in Table 4.1. For core dynamics studies (**IV**), some of the tested polyplexes were prepared with FITC-labelled DNA and Cy3-labelled BPEI or PLL (**IV**: Fig. 2).

Table 4.1 *Properties of cationic carriers used in Studies I–VI.*

Cationic carrier	M_W [1] (kDa)	Amine density ((Da)$^{-1}$)	N_N [2]	Order of amines[3]	N/P ratio	Publication
Polymeric						
SPEI	0.7	(43)$^{-1}$	16.3	1º, 2º, 3º	0.2–8	I, VI
LPEI	22	(43)$^{-1}$	511.6	2º	0.2–8	I, III, VI
BPEI	25	(43)$^{-1}$	581.4	1º, 2º, 3º	0.2–8	I–VI
PLL	200	(128)$^{-1}$	1562.5	1º	0.2–8	III, IV
PBAEs						
F28	16.1	(301)$^{-1}$	53.5	3º	1.1–108.0	II
C36	21.2	(315)$^{-1}$	67.3	3º	1.0–103.2	II
D24	9.5	(515)$^{-1}$	18.5	3º	0.6–63.1	II
E28	14.3	(315)$^{-1}$	45.4	3º	1.0–103.2	II
U28	15.6	(403)$^{-1}$	38.7	3º	0.8–80.7	II
C28	27.9	(287)$^{-1}$	92.7	3º	1.1–113.2	II
AA24	8.1	(437)$^{-1}$	18.4	3º	0.7–62.5	II
AA28	20.9	(435)$^{-1}$	48.0	3º	0.8–74.7	II
C32	18.1	(301)$^{-1}$	60.1	3º	1.1–108.0	II
JJ28	16.8	(301)$^{-1}$	55.8	3º	1.1–108.0	II
Lipidic						
DOSPER	1.09	(272)$^{-1}$	4	1º, 2º	1.2 –12	VI

[1] M_W average molecular weight of polymer (g/mol=Da), [2] N_N average number of amine groups per carrier molecule, [3] the amino groups involved in binding the DNA which are taken into account for calculating N/P ratios.

Table 4.2 *Expression plasmids.*

Plasmid type[1]	Size (kb)	Promoter	Reporter gene	Publication
pCMVβ	7.2	CMV	β-galactosidase	I–III, V
pCLuc4	5.2	CMV	luciferase	IV, V
pTKBPVlacZ	16.2	RSV LTR	β-galactosidase	VI

[1]All plasmids were produced in and isolated from Escherichia coli using ion-exchange columns, pDNA supercoiled topology was confirmed by agarose gel electrophoresis. The concentration and purity of pDNA were determined by absorption measurements at 260 and 280 nm.

CMV: cytomegalovirus; RSV LTR: Rous sarcoma virus long terminal repeat.

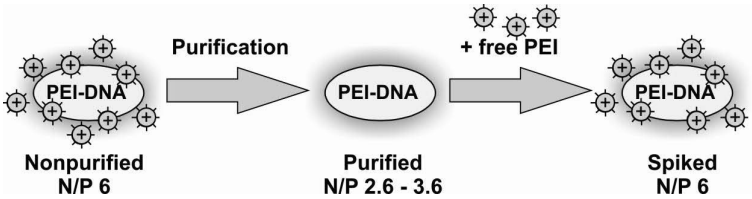


Figure 4.1 *Terms "Nonpurified", "Purified ", and "Spiked " used for the polyplexes in Studies I and V and their N/P ratios are presented.*

4.2 Physicochemical characterization (III, V)

To qualitatively demonstrate changes in particle size, the mean hydrodynamic diameters of polyplexes were determined by a dynamic light scattering (DLS) technique at 90º scattering angle using a Malvern Zetasizer auto plate sampler (**III**). Tested series of BPEI, LPEI, and PLL polyplexes with N/P ratios in the range of 0.4-8 were prepared by stepwise method in 50 mM MES/50 mM HEPES/75 mM NaCl buffer at pH 5.2 and 7.4.

To investigate the absolute size and shape of particles, multi-angular static light scattering (SLS) experiments were conducted at a larger angular range (**V**). Different BPEI polyplex formulations in 20 mM HEPES/5 mM NaCl, pH 7.4 were measured with the light scattering technique using a Brookhaven Instruments BIC-200 SM goniometer, a BIC-TurboCorr digital pseudo-cross-correlator, and a BI-CrossCorr detector including two BIC-DS1 detectors. A Coherent Sapphire 488–100 CDRH laser operating at wavelength 488 nm (10–50 mW output power) was used as the light source. Distributions of the hydrodynamic sizes were collected at scattering angles between 40° and 140°, and the data analysis was performed according to Andersson et al. (2004). For the estimation of surface charge, zeta-potential was measured with a Zetasizer Nano-ZS ZEN3600. All measurements were performed at 20 °C.

4.3 Fluorescence measurements (I–IV)

The formation of polyplexes was studied by time-resolved fluorescence spectroscopy using ethidium bromide (ETI) as a fluorescence probe (**I–III**). The change of ETI fluorescence properties during polyplex formation was used for monitoring the equilibrium between DNA and various cationic polymers (Figure 4.2).

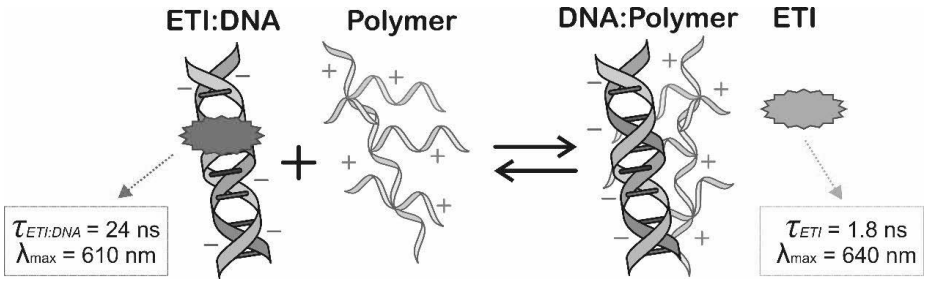


Figure 4.2 *Schematic picture of the changes in ETI fluorescence during polyplex formation. Free ETI has a weak fluorescence signal at 640 nm, but when intercalates between A-T base pairs of DNA its fluorescence maximum shifts to 610 nm and the quantum yield increases by about 7–8 times. Due to the ETI–DNA complexation, the fluorescence lifetime of ETI increases from 1.8 ns in a solution to 24 ns in the presence of DNA. Conformational changes in DNA after a cationic polymer induced condensation displaces ETI from DNA, thus decreasing fluorescence lifetime from 24 ns in DNA back to 1.8 ns in solution.*

Polyplexes for fluorescence measurements were prepared in 50 mM MES/50 mM HEPES/75 mM NaCl buffer at pH 5.2 and 7.4, and the final DNA concentration was adjusted to 300 μM per nucleotide. In all experiments, an ETI:nucleotide mole ratio of 1:15 was used to ensure that all ETI in the initial solution was bound by DNA (i.e. without polymer, there was no free ETI in the system). The time-resolved fluorescence was measured by a time-correlated single photon counting (TCSPC) system (PicoQuant GmBH) consisting of a PicoHarp 300 controller and a PDL 800-B driver. The samples were excited with the pulsed diode laser head LDH-P-C-485 at 483 nm with a 130 ps time resolution. The signals were detected with a microchannel plate photomultiplier tube (Hamamatsu R2809U). To diminish the influence of the scattered excitation, a cut-off filter was used in front of the monitoring monochromator. To study the time-resolved spectra, the decays were collected using a constant accumulation time (300 s) in the 560–670 nm wavelength range with 10 nm increments. The decays were simultaneously fitted to the sum of two exponents in equation (1):

$$I(t,\lambda) = a_1(\lambda)e^{-t/\tau_1} + a_2(\lambda)e^{-t/\tau_2} \qquad (1)$$

where τ_i is the global lifetime and $a_i(\lambda)$ is the local amplitude at a particular wavelength. The amplitudes $a_i(\lambda)$ represent the decay-associated spectra (DAS), which in the case

of a mixture of different non-interacting fluorescing species corresponds to the individual spectra of the species (ETI–DNA complex and ETI free in solution). The local amplitudes, $a_i(\lambda)$, were corrected depending on the sensitivity of the detector at different wavelengths. The corrected spectral areas (A_i) were calculated as $A_i = \int \alpha_i(\lambda) d\lambda$.

During the studies it became evident that more precise data are obtained if the raw spectral areas are corrected to the relative quantum yield (**III**). Because the fluorescence quantum yield of free ETI in a bulk solution is lower that the yield corresponding to ETI–DNA complex, the relative fluorescence quantum yield (ϕ_{rel}) was calculated from the steady-state absorption (UV-VIS spectrophotometer Schimadzu UV-3600) and fluorescence (Fluorolog Yobin Yvon-SPEX, $\lambda_{exc} = 483$ nm) spectra according to equation (2):

$$\phi_{rel} = \frac{\phi_{ETI}}{\phi_{ETI-DNA}} = \frac{I_{ETI}}{I_{ETI-DNA}} \cdot \frac{A_{ETI-DNA}}{A_{ETI}} \qquad (2)$$

where ϕ_{ETI} is the quantum yield of free ETI, $\phi_{ETI-DNA}$ is the quantum yield of the ETI–DNA complex, I_i is the area of the fluorescence spectra with an excitation wavelength of 483 nm, and A_i is the absorbance at a wavelength of 483 nm. The quantum yield corrected spectral area for the short-living component was calculated as $A_{1,QY} = A_1/\phi_{rel}$. As the polymer is added to the ETI–DNA complex, the polymer (P) binds DNA and ETI is freed into the bulk solution: $ETI{:}\,DNA + P \rightleftarrows P{:}\,DNA + ETI$. During the polyplex formation ETI fluorescence lifetime changes from 24 ns for ETI–DNA complex back to 1.8 ns for free ETI. Therefore, in the presence of polymer there was a two-exponential fluorescence decay curve with two distinct fluorescence lifetimes corresponding to ETI–DNA complex and free ETI. The proportion of the short-living decay component, B, corresponding to free ETI in the solution was calculated from the spectral areas of the components as follows:

$$B = \frac{A_{1,QY}}{A_{1,QY} + A_2} \times 100\% \qquad (3)$$

where A_2 is the spectral area of the long-living component (ETI–DNA complex). Because the amount of free ETI in a solution is directly proportional to the amount of forming polyplex (or the fraction of DNA bound to polymer), the extent of polyplex formation was assessed by monitoring the relative amount of free ETI fluorescence as a function of N/P ratio. The cooperativity and binding constants for the polyplex formation were calculated using independent and cooperative binding models as described in Study **III**.

It should be noted that the fluorescence measurements of BPEI, SPEI, and LPEI polyplexes in Study **I** were done in a 50 mM MES/50 mM HEPES/75 mM NaCl solution of pH 9.2, not pH 7.4, as mentioned in the publication's experimental section (purified, spiked, and control BPEI samples were measured at pH 7.4). As the pH difference might affect the binding constants, the measurements were repeated at pH 7.4 (full experimental data were only obtained for BPEI and LPEI samples), and the collected data were analysed as explained above.

The structural dynamics in BPEI and PLL polyplexes (**IV**) were studied by steady-state fluorescence microscopy and double labelling based fluorescence resonance energy transfer (FRET) techniques using FITC-labelled DNA as the donor and Cy-3 labelled polymers as acceptors, as described in more detail in Study **IV**.

4.4 Transfection experiments (I, III, V, VI)

All transfection experiments were performed in adherent cell culture monolayers. Cell cultures used for in vitro transfections are listed in Table 4.3. All cell lines were passaged twice a week and seeded into well-plates 24 h prior to the experiments to reach 70–80% confluency on the day of transfection. Transfections were carried out in serum-free conditions and DNA complexes were added dropwise to wells and incubated with cells for 4 h (**VI**) and 5 h (**I, III, V**).

Table 4.3 *Cell cultures.*

Cell line	Cell type	Tissue origin	Species	GAG biosynthesis	Publication
SMC	smooth muscle cells	aorta	rabbit	normal	I, VI
CV1-P	fibroblasts	kidney	monkey	normal	I, III
ARPE-19	epithelial	retina	human	normal	III
CHO	epithelial	ovary	hamster, Chinese	normal	I, III, V
pgsB-618	epithelial, galactosyltransferase I-deficient mutants	ovary	hamster, Chinese	HS- and CS-deficient	V
pgsD-677	epithelial, heparan sulfate polymerase-deficient mutants	ovary	hamster, Chinese	HS-deficient, but accumulates CS	V

4.4.1 Beta-galactosidase expression

The levels of active beta-galactosidase enzyme (β-gal) expressed in the transfected cells were assayed 48 h post-transfection from cellular lysates using ONPG assay. The β-gal activity was quantified spectrophotometrically at 405 nm wavelength using ONPG as the substrate. Purified β-gal was used as the reference standard, and cell lysates from non-transfected cells were used as a correction for endogenous levels of cellular β-gal. The β-gal activity was normalized to the total amount of protein in the cell lysates (mU/mg of protein). Protein content was quantified by Bradford assay using bovine serum albumin as the standard. Histochemical staining with X-gal substrate was used to characterize and trace β-gal expression at single-cell level (**VI**). The β-gal activity was detected 24 h post-transfection from paraformaldehyde-fixed cell monolayers that were photographed and the number of blue-coloured cells was counted.

4.4.2 Luciferase expression

The levels of expressed luciferase were quantified 48 h post-transfection from cell lysates by measuring light emission on a luminometer using standard luciferase assay reagent (20 mM tricine, 1.07 mM $(MgCO_3)_4$ $Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, pH 7.8, 2 µM coenzyme A, 470 µM luciferin, and 530 µM ATP). The luminescence was normalized to the protein content of each sample (RLU/mg of protein) that was determined by Bradford assay using bovine serum albumin as the standard.

4.4.3 Toxicity assay

The immediate cytotoxicity mediated by DNA complexes was studied by lactate dehydrogenase (LDH) fluorometric assay (CytoTox-ONE™ Homogenous Membrane Integrity Assay, Promega), which measures the release of intracellular LDH into the cell culture medium as an indication of cell membrane damage. The cell culture media samples were collected 5 h after transfection, and the levels of released LDH from damaged cells were measured by supplying lactate, NAD^+, and resazurin as the substrates in the presence of diaphorase.

4.4.4 Intracellular elimination of pDNA

For cellular elimination studies, the amount of cell-associated pDNA was determined from the whole-cell samples that were collected every 24 h up to 5 days after transfection. Quantification of the amount of pDNA expressing luciferase gene was performed with the quantitative real-time PCR (qRT-PCR) method according to Ruponen et al. (2009) and employing an ABI Prism 7000 sequence detection system (Applied Biosystems). Prior to analysis, each sample at 1:200 dilution was treated with heparan sulfate (9 µM) in order to release or relax pDNA from PEI, which was followed by further dilution in sterile water to the final dilution ratio of 1:2000 (volume/volume). For qRT-PCR analysis, the diluted sample (5 µl) was mixed with PCR reagent mixture (10 µl) containing SYBR® Green Master Mix, forward (5′-GGC GCG TTA TTT ATC GGA GTT-3′) and reverse (5′-TAC TGT TGA GCA ATT CAC GTT CAT T-3′) primers towards luciferase encoding plasmid. The size of the amplification product was 73 bp. PCR cycle conditions were 50 °C for 2 min, 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Individual calibration curves were generated separately for each experiment containing 10–2500 pg of pDNA in the presence of cell lysates.

5 Results

5.1 Formation of polymer–DNA polyplexes (I–IV)

The mechanisms of polyplex formation were studied by comparing DNA-complexation behaviour of branched and linear PEI, PLL and PBAEs. Initially, the formation of DNA polyplexes was investigated by a time-resolved fluorescence spectroscopy method as proposed in Vuorimaa et al. (2008). However, as the thesis studies progressed, it became evident that the cooperative binding model describes the forming polyplex more accurately (**III**: Fig. 6, Fig. S4) than the independent binding model used at the beginning of this project (**I, II**). The independent binding model, in contrast to the cooperative binding model, does not consider the simultaneous or subsequent binding of other amine ligands at other unoccupied phosphate binding sites on DNA. Therefore, the relevant data from **I** and **II** was reanalysed with the cooperative binding model for multivalent binding ligand to multiunit substrate and the revised binding isotherms together with binding constants are presented in this section. Although the published results at the very beginning of the studies (**I, II**) were obtained with an independent binding model, they show the correct trend and provided a framework for subsequent studies (**III, IV**).

The fluorescence measurements were performed with polyplexes prepared with a stepwise method. Compared to a direct method where all polyplexes are assayed after the same incubation time, the stepwise method affects the age of the polyplex. Due to the length of fluorescent measurements, the age of the initial polyplex sample increases with increasing N/P ratios. However, direct comparison of BPEI polyplexes prepared by the two methods did not reveal any major differences in the fluorescence lifetimes (**I**: S1), DAS (**I**: S3) or in a plot of A_c^2/A_c^1 ratio against the inverse concentration of polymer (**I**: S4). It could be assumed, therefore, that the preparation method has a negligible effect on the overall formation of polyplex.

5.1.1 Binding constants for polyplex formation

The polyplex formation was monitored by plotting B (proportion of DNA bound by the polymer) as a function on N/P ratio at pH 7.4. The results showed that for all tested polymers, the proportion of bound DNA clearly increased with increasing N/P ratio until it reached saturation. The sigmoidal shape of the curves indicates that the initial binding of a polymer to DNA promoted the stronger binding of another polymer molecule.

The binding isotherms for BPEI, LPEI and PLL displayed similar behaviour and reached a saturation limit close to 100% at an N/P ratio of 2, indicating polyplex formation (**III**: Fig. 2). This saturation point also corresponded to a sharp increase in the size of polyplexes and an increase in the polydispersity index (PDI) of samples observed during particle size measurements (**III**: Fig. 3). While PEIs and PLL

saturated near 100%, most of the PBAEs had saturation levels close to 80%; the values of saturation limits were 73% for F28 and 91% for JJ28 (Figure 5.1). The turning points of the binding isotherms were close to an N/P ratio of 22. The differences in binding isotherms observed within PBAE series were small, except for the polymers C36 and F28, which displayed an unexpectedly lower degree of DNA condensation.

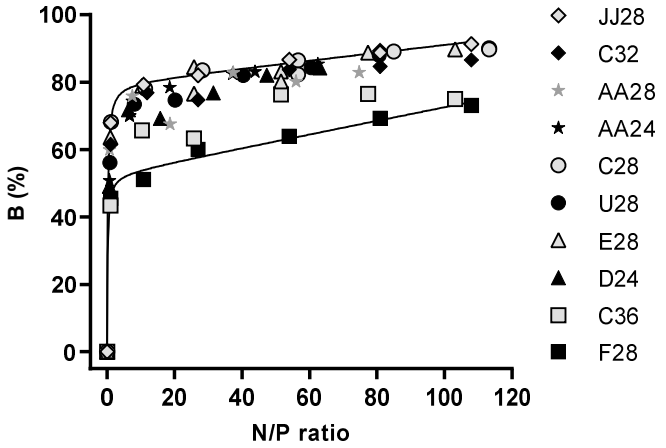


Figure 5.1 *Binding isotherms for PBAE polymer series. Fraction of DNA bound by polymer (B) as a function of polymer amine group concentration.*

Effect of polymer

To compare the DNA condensation behaviour between polymers, the cooperativity and binding constants for polyplex formation were estimated according to the Hill plot equation for multivalent ligands binding to multisubunit substrates. The results are summarized in Table 5.1.

The Hill plots for BPEI, LPEI and PLL appeared fractional, as composites of three lines with different slopes (α) (**III**: Fig. S3a, Fig. 5a, Fig. S3c, respectively), which suggests a change in the DNA binding mechanism with increasing N/P ratio. BPEI and PLL displayed similar DNA binding behaviour changing from independent binding without cooperativity (at N/P ≤0.6) to positive cooperativity (0.6≤ N/P ≤ 2), as judged from the Hill coefficients. By contrast, the Hill plots for LPEI were associated with slightly negative (at N/P ≤0.6) and positive (0.6≤ N/P ≤2) cooperativity phases. At N/P ≥2, the fraction of bound DNA reached a maximum and additional binding of either of the polymers did not occur. Therefore, the polyplex core formation for both PEIs and PLL was completed at the same N/P ratio of 2.

For PBAEs, the Hill plots showed either a single straight line over all polymer concentrations with α-values smaller than 0.5, indicating strong negative cooperativity (AA24, C28, C36), or biphasic lines with various slopes (α) (Table 5.1), indicating negative cooperativity at low N/P ratios and independent or slightly positive cooperativity at high N/P ratios. The turning points of the Hill plots were in an N/P ratio range of 15.8–27.

In general, the results showed that the higher the overall cooperativity binding constant, the lower the N/P ratio required for the saturation of the system and the formation of the polyplex core. Overall cooperativity binding constants differed between polymers in the order PEIs>PLL>>PBAEs (Table 5.1). For PEIs, which displayed positive cooperativity, the values of overall binding constants were five orders of magnitude higher than those for PBAEs, which displayed no (or very low) cooperativity. The overall cooperativity binding constant $(K_{co})^{\alpha}$ values obtained for both PEIs and PLL (10^8–10^9 M^{-1}) were higher than the corresponding average cooperative binding constant per amine group K_{co} values (10^3 M^{-1}). Binding constant values for PBAEs, showed the opposite trend, due to negative cooperativity during PBAE polyplex formation, $(K_{co})^{\alpha}$ values were lower than K_{co} values. For PLL, the $(K_{co})^{\alpha}$ values were lower than those of PEIs, however, the K_{co} values were always higher than for PEIs.

Effect of environmental pH

The binding affinity between polymer and DNA was investigated at two different pH values corresponding roughly to extracellular pH of healthy tissues (pH 7.4) and endocytic vesicles (pH 5.2). A comparison of the results measured at pH 7.4 with those at pH 5.2 clearly showed that pH has an influence on the PEI and PLL polyplex formation. The Hill plots at pH 5.2, which were composites of only two lines with steeper slopes (α) than for pH 7.4, indicated a higher degree of cooperativity even at the lowest polymer concentrations (**III**: Fig. 5b, Fig. S3b,d). The cooperative binding affinity of both PEIs and PLL significantly increased with decreasing pH of the buffer (**III**: Table 1). The higher binding affinities at lower pH resulted in smaller sized polyplexes (**III**: Fig. 3). The values of overall cooperative binding constants $(K_{co})^{\alpha}$ at pH 5.2 were in the range of 10^9–10^{14} M^{-1} and followed the same order BPEI>LPEI>PLL as at pH 7.4. However, the average cooperative binding constants per amine (K_{co}) were lower at pH 5.2 than at pH 7.4. The values were in the range of 10^3 M^{-1} and followed the order PLL>BPEI>LPEI, which is the same as at pH 7.4. This difference was caused by smaller amounts of positive species at pH 7.4, leading to a lower cooperativity at pH 7.4 than at pH 5.2.

Table 5.1 *Cooperative binding constants for PEI, PLL and PBAE polyplexes at pH 7.4.*

Polymer	N/P ratios	α	Cooperativity*	$(K_{co})^{\alpha}$ (M^{-1})	K_{co} (M^{-1})
BPEI	0.2–0.6	0.97	independent	5.7×10^3	7.7×10^3
	0.6–2.0	2.31	positive	5.4×10^8	6.1×10^3
LPEI	0.2–0.6	0.76	negative	7.5×10^2	6.3×10^3
	0.6–2.0	2.32	positive	4.8×10^8	5.5×10^3
PLL	0.2–0.6	1.01	independent	1.1×10^4	1.01×10^4
	0.6–2.0	2.06	positive	9.7×10^7	7.4×10^3
PBAE - F28	1.1–27.0	0.17	negative	3.06	8.48×10^2
	27.0–108.0	0.73	negative	49.1	2.07×10^2
PBAE - C36	1.0–103.2	0.35	negative	13.1	1.55×10^3
PBAE - D24	0.6–15.8	0.29	negative	12.6	6.28×10^3
	15.8–63.1	1.46	positive	5.26×10^3	3.61×10^3
PBAE - E28	1.0–25.8	0.27	negative	16.7	2.89×10^4
	25.8–103.2	0.96	independent	4.20×10^2	5.57×10^2
PBAE - U28	0.8–20.2	0.27	negative	13.0	1.13×10^4
	20.2–80.7	1.35	positive	3.26×10^3	4.05×10^2
PBAE - C28	1.1–113.2	0.35	negative	32.9	1.94×10^4
PBAE - AA24	0.7–62.5	0.46	negative	48.7	4.40×10^3
PBAE - AA28	0.7–18.7	0.10	negative	3.54	2.34×10^5
	18.7–74.7	0.86	negative	2.55×10^2	6.11×10^2
PBAE - C32	1.1–27.0	0.21	negative	9.48	3.54×10^4
	27.0–108.0	0.82	negative	1.69×10^2	5.36×10^2
PBAE - JJ28	1.1–27.0	0.24	negative	14.7	7.54×10^4
	27.0–108.0	1.16	independent	1.27×10^3	4.82×10^2

α: experimental Hill's cooperativity coefficient; *indicated binding cooperativity; $(K_{co})^{\alpha}$: overall cooperative binding constant for the reaction $DNA + nP \rightleftharpoons DNA\text{-}Pn$; K_{co}: average cooperative binding constant for the binding of one functional amine group according to the reaction $DNA\text{-}P_{X-1} + P \rightleftharpoons DNA\text{-}P_X$ $(X = 1, 2, \dots, n)$.

5.1.2 Effect of binding constant on transfection efficiency

The efficiency of PBAE–DNA complex formation can to some extent be estimated from the saturation levels obtained from the binding isotherms. However, only small differences in the *B* values for PBAEs were observed, although for C36 and F28 the increase was the lowest (Figure 5.2). Therefore, the relative efficiency of DNA complexation by PBAEs (**II**: Fig. 5) was estimated as the difference in the ratio of the maximum amplitudes $a_{i,max}$ at each N/P ratio (**II**: Eq. 2) and calculated with N/P ratio of 50, which has been shown to be effective in transfections. The increased

nanoparticle formation for most of the tested PBAEs was found to positively correlate with increased in vitro transfection efficiency (**II**: Fig. 6).

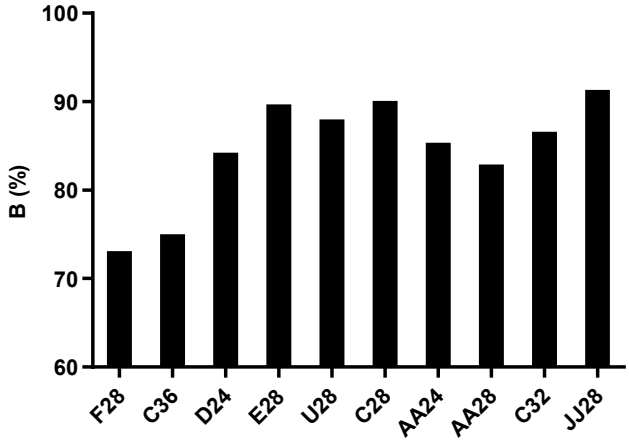


Figure 5.2 *Saturation (B-value) differences between PBAE polymer series.*

When comparing the overall binding constants $(K_{co})^{\alpha}$ of PBAEs with their transfection efficacies, no clear correlation was observed (Figure 5.3), as similar binding constants resulted in variable transfection outcomes. According to Figure 5.3 PBAEs could be roughly divided into three groups with small ($<10^2$ M^{-1}), intermediate (10^2–10^3 M^{-1}), and high ($>10^3$ M^{-1}) binding constants. Although binding constants for PBAE–DNA polyplexes were much smaller than for BPEI (10^8 M^{-1}), all PBAEs polyplexes exhibited transfection efficiency superior to BPEI (Anderson et al., 2005). The most effective transfection polymers C32, JJ28, and C28 belong to the group with intermediate, high, and small binding constant, respectively. These PBAEs share a common structure and differ by only a single carbon atom in their amine (C32 and C28) or diacrylate (JJ28 and C28) monomer carbon chains (**II**: Table 1). When the structures of the diacrylate and amine monomers were varied, the binding affinities had a tendency to change as follows. By increasing the length of the amine monomer chain by one carbon (C28→C32), both the nanoparticle formation efficiency and $(K_{co})^{\alpha}$ increased, which were associated with increased transfection efficiency. Increasing the length of the diacrylate monomer by one carbon (C28→JJ28) also resulted in higher $(K_{co})^{\alpha}$ and nanoparticle formation efficiency, again associated with improved transfection. However, a further increase in amine (C32→C36) or diacrylate (JJ28→E28) monomer lengths had the completely opposite effect. The $(K_{co})^{\alpha}$ values decreased and the nanoparticle formation efficiency suddenly dropped to lower values than for C28, which was associated with diminished transfection efficacy (Figure 5.4). Other polymers synthesized from amine monomer 28 and structurally unique diacrylates displayed (i) small $(K_{co})^{\alpha}$ and low transfection efficacy (F28), (ii) both intermediate $(K_{co})^{\alpha}$ and transfection efficacy (AA28), and (iii) high $(K_{co})^{\alpha}$ and medium levels of transfection (U28).

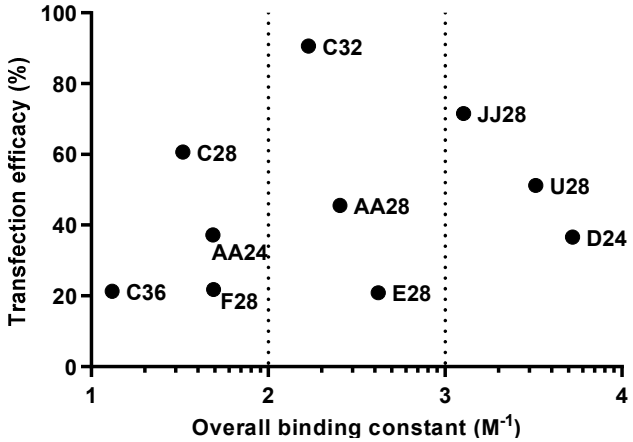


Figure 5.3 *Effect of overall cooperative binding constant on the in vitro transfection efficacy of PBAE polymer series. The PBAEs with small (<10^2 M^{-1}), intermediate (10^2–10^3 M^{-1}) and high (>10^3 M^{-1}) overall binding constants are divided with dotted lines.*

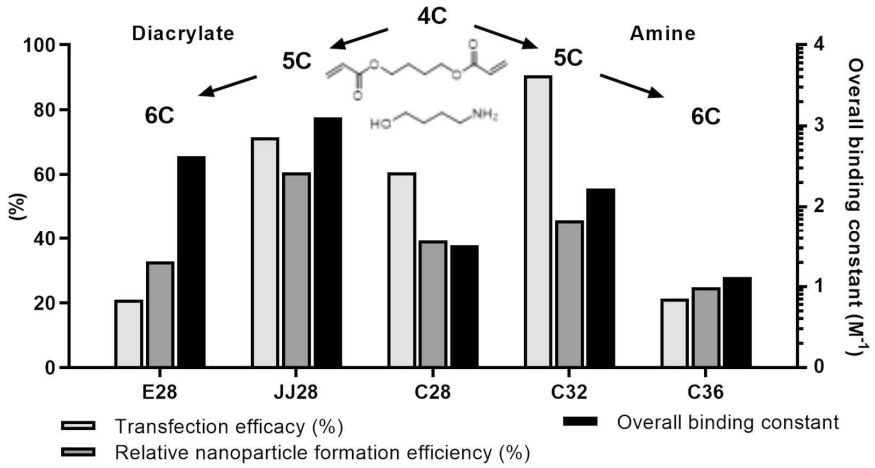


Figure 5.4 *Example of single carbon atom difference in PBAE structure and its effect on transfection efficacy, the relative nanoparticle formation, and the overall cooperative binding constant. Polymers E28, JJ28, C28 differ in their amine monomer carbon chains whereas polymers C28, C32, C36 differ in their diacrylate monomer carbon chains.*

When the overall cooperative binding constants for PEIs and PLL obtained from the positive cooperativity phase (**III**: Fig. S5a) were analysed with transfection efficacies in three different cell lines (**III**: Fig. S5b), some similarities were observed. Both binding constants and transfection efficiencies of PEIs were much higher than those of PLL. Although BPEI formed nanoparticles more effectively than LPEI, its ability to transfect all cell lines was considerably lower than that of LPEI.

It is noteworthy that the binding constant reflects only the formation of the polyplex core, which was completed in PEIs at approximately N/P ~2. In cell

transfections, however, polymer–DNA complexes are usually used at much higher N/P ratios (PEI at N/P of 6–8, PLL at N/P of 2–4, PBAEs at N/P of 40–60). At such high N/P ratios, all ETI molecules are totally displaced by the DNA-condensing polymer into a bulk solution, and therefore, ETI cannot reflect any changes to the polyplex occurring after core formation.

5.1.3 Dynamics of polymer–DNA nanoparticles

In core–shell dynamics studies, DNA and polymeric vectors were individually labelled with FITC and Cy3 dye, respectively, as a donor and acceptor pair for the fluorescence resonance energy transfer (FRET). In contrast to the TCSPC method using ETI dye, the FRET-based method enabled a detection of changes in the polyplex state after the core formation, i.e. at N/P >2. The results confirmed that polyplexes can be described as core–shell structures. In general, a very strong and stable nanoparticle core was formed at N/P ratio of 2. Such a core was surrounded by a loose, positively charged shell when an extra amount of polymer was added. For PEI polyplexes formed at N/P >2, there was a clear exchange of PEI molecules between the nanoparticle core and the shell, as indicated by changes in excitation spectra (**IV**: Fig. 5d) and the Stern–Volmer plots (**IV**: Fig. 5f). However, this kind of behaviour did not occur for PLL polyplexes, as Cy3-labelled PLL in the shell did not interact with the DNA in the nanoparticle core at all N/P ratios tested (**IV**: Fig. 5c, Fig. 5e).

5.2 Free PEI in polyplex formation and gene delivery (I, III, V)

5.2.1 Effect of free PEI on physicochemical properties of polyplexes

The effect of free polymer chains in the polyplex solution was studied by comparing properties of BPEI polyplexes with and without free PEI. It is evident that direct preparation of stable polyplexes only with PEI chains bound to DNA is impossible because the complete condensation of DNA that occurs at N/P ~2 is accompanied by substantial particle aggregation (**III**: Fig. 3a,b). However, the removal of excessive PEI chains from polyplexes by size exclusion chromatography or electrophoresis resulted in stable polyplexes with an N/P ratio close to 3 (**I**, **V**).

Particle size and surface charge of polyplexes were almost unchanged during the purification (**V**: Table 1). For polyplexes with free PEI, the theoretically derived molecular shape parameter ($R_g/R_h = 0.71$), which provides information on the shape of nanoparticles, suggests that the particles are spherical, denser in the vicinity of their centre of gravity. This indicates that polyplexes have a core–shell structure. Most probably the hydrophobic core is formed by DNA/PEI entangled complexes and the hydrophilic solvent-draining outer shell is formed by excess PEI on the surface. Removing free PEI chains from the polyplex leads to an increase of the R_g/R_h ratio to

0.9, correlating with the more loosely packed, flowing-through structure. Supplementing purified polyplexes with free PEI back to the original N/P ratio of 6 restored all of the values of physicochemical properties to practically the same levels as for nonpurified polyplexes (**V**: Table 1). Results from light scattering were supported by time-resolved fluorescence spectroscopy measurements. The TCSPC method showed that polyplex properties do not significantly change during purification and spiking and retain the structure formed at the original N/P ratio (**I**: Table 2, S7 and S8).

5.2.2 Effect of free PEI on transfection efficiency and toxicity

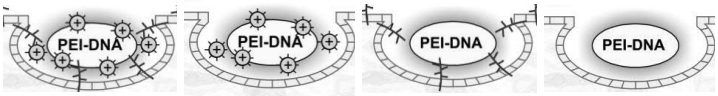
Initially, β-galactosidase gene expression of polyplexes with and without free PEI chains was evaluated in three different cell lines (CV1-P, SMC, and CHO) rich in GAG content. The removal of free PEI from polyplexes resulted in a sharp decrease of transgene expression in all cell lines tested (**I**: Fig. 7); the transfection efficiency was reduced 5.5-fold in CV1-P, 9.9-fold in SMC, and 5.2-fold in CHO cells. When the extra PEI (equivalent to the amount of free PEI in nonpurified polyplexes) was added to purified polyplexes, the transgene expression returned close to the levels for nonpurified polyplexes. This indicates that free PEI is essential for transfection of cells with high GAG content.

To study whether the effect of free PEI on transfection efficacy is related to the interaction with cell-surface GAGs, transfection experiments were also performed with genetically modified CHO cells with different GAG expression profiles (Esko et al., 1985). GalTI$^-$ and HSPol$^-$ CHO mutants offered a good model because of significant variations of cell-surface GAGs. Compared to CHO wild-type cells (0.7×10^3 GAG chains/cell), GalTI$^-$ mutants lack galactosyltransferase I required for the assembly of the core protein, and thus, nearly all cell-surface GAGs are absent (only 40 GAG chains/cell) (Ruponen et al., 2004). HSPol$^-$ mutants, which are defective in HS biosynthesis, lack HS but accumulate large amounts of CS. Due to the accumulation of CS, the total amount of cell-surface GAGs (1.9×10^3 GAG chains/cell) is nearly 3-fold higher than in wild-type cells (Ruponen et al., 2004).

Wild-type and mutant cells were transfected with purified, nonpurified and spiked polyplexes at DNA concentrations in the 2–16 μg/ml range and evaluated for transgene expression (**V**: Fig. 1) and cellular toxicity (**V**: Fig. 2). Since at the lowest DNA concentration the transgene expression was negligible, Table 5.2 only summarizes results of nonpurified and purified polyplexes at DNA concentrations of 4–16 μg/ml. In general, the transgene expression of nonpurified polyplexes (with free PEI), which increased dose-dependently, was similar in all cell lines tested regardless of GAG content. Only in GalTI$^-$ cells, lacking nearly all cell-surface GAGs, transfection levels were 3.5-fold higher at a DNA concentration of 8 μg/ml (Table 5.2). However, different behaviour was observed for purified polyplexes (without free PEI); in the presence of cell-surface GAGs (wild-type and HSPol$^-$ cells), transfection efficiency was minimal and remained exceptionally low despite increased polyplex quantities. The cytotoxicity of purified polyplexes was very low and

remained constant at all DNA concentrations tested, whereas the cytotoxicity of nonpurified polyplexes gradually increased up to 4.1-fold in wild-type and 5.4-fold in HSPol$^-$ cells.

Table 5.2 *Effects of cell-surface GAGs and free PEI on non-viral gene delivery.*

Polyplex		Nonpurified			Purified		
CHO cells		Wild-type	HSPol$^-$	GalTI-	Wild-type	HSPol$^-$	GalTI-
	DNA (µg/ml)						
Transgene expression	4	1.00	2.67	15.51	0.57	0.66	3.41
	8	8.54	8.95	29.95	1.65	0.75	16.53
	16	11.98	12.73	11.05	1.61	0.78	27.12
Cellular toxicity	4	1.00	0.76	1.31	0.73	0.70	0.99
	8	1.38	1.77	4.47	0.72	0.85	1.73
	16	2.83	4.62	14.81	0.70	0.85	1.96
Intracellular exposure	-	1.00	1.56	0.64	1.45	2.23	4.82

Summary of the results from transgene expression, cellular toxicity, and intracellular exposure of cells to pDNA experiments. All values are normalized to a transfection with nonpurified polyplexes at 4 µg/ml DNA in wild-type cells. Nonpurified: with free PEI, purified: without free PEI.

Interestingly, in the absence of cell-surface GAGs (GalTI$^-$ cells) transfection efficiency of purified polyplexes increased in a concentration-dependent manner. At the highest DNA concentration, purified polyplexes were 2.5-fold more efficient than nonpurified polyplexes. It seems that not the lack of free PEI but the inhibition by GAGs may have attributed to very low gene expression of purified complexes in wild-type cells. There is a clear possibility that this difference could also be attributed to the higher toxicity of free PEI (7.5-fold), which causes less transfected cells to survive, thus lowering the transgene expression of nonpurified polyplexes. Therefore, additional transfection experiments in GalTI$^-$ cells were performed with luciferase reporter gene and normalized to cellular toxicity to discriminate the influence of different cellular toxicities of purified and nonpurified polyplex from their actual transfection capacity. Unlike for nonpurified polyplexes, transfection efficiency of purified polyplexes markedly increased with an increasing dose of purified polyplexes (**V**: Fig. 3). At the highest DNA concentration tested, transgene expression of nonpurified polyplexes was again significantly lower (2.6-fold) and correlated well with the results observed with beta-galactosidase reporter gene (**V**: Fig. 1, GalTI$^-$). This result indicates that the free PEI toxicity in nonpurified polyplexes was not the main cause of the observed difference in transfection efficiency.

5.2.3 Effect of free PEI on cellular elimination of pDNA and significance of cell-surface GAGs

The role of interactions between free PEI chains and cell-surface GAGs on the intracellular elimination of pDNA was studied in both wild-type and mutant CHO cells transfected with purified, nonpurified and spiked polyplexes. It is evident from the results shown in **V**: Fig. 4 that the intracellular elimination of pDNA was slow in all cell lines and was unaffected by the presence of free PEI chains. Therefore, it is unlikely that the improvement seen in transfection is related to the elimination of DNA from the cells. Nevertheless, the intracellular elimination was much faster in wild-type cells (**V**: Fig 4A) than in HSPol$^-$ and GalTI$^-$ mutants (**V**: Fig. 4B,C). The presence of cell-surface heparan sulfate may explain the faster elimination rates, presumably arising from the fact that the internalized polyplexes bound to heparan sulfate may follow the lysosomal degradation pathway.

In GAG-deficient cells (GalTI$^-$), purified polyplexes showed clearly higher cell association than nonpurified polyplexes throughout the experiment (**V**: Fig. 4C). At 48 h after transfection, when the transgene expression reached its maximum, the difference was over 10-fold. The addition of free PEI to purified polyplexes reduced the amount of cell-associated pDNA close to the levels of nonpurified polyplexes. Different behaviour was observed in GAG-containing cells (wild-type and HSPol$^-$). The amounts of cell-associated pDNA delivered by purified polyplexes were only slightly increased or at the same level as for nonpurified polyplexes. Although the cell association of purified polyplexes did not markedly differ from nonpurified polyplexes, their gene expression was significantly reduced (**V**: Fig. 1).

The intracellular exposure of all cell lines to pDNA after the transfection with purified polyplexes was much higher than with nonpurified polyplexes (**V**: Table 2; Table 5.2), especially in GAG-deficient cells, where the increase was 7.5-fold. However, after addition of free PEI, the initial high values dropped to lower levels, similar to nonpurified polyplexes. This indicates that free PEI decreases the quantity of cellular pDNA.

5.3 Effect of PEI structure on lipopolyplex-mediated gene delivery (VI)

The effect of PEI structure on gene delivery synergism between PEI polyplexes and cationic liposomes was studied by comparing transfection efficiencies of various lipopolyplex (LPP) formulations formed with linear or branched PEI and multivalent cationic lipid DOSPER.

LPEI–LPPs were prepared by sequential addition of DOSPER liposomes at mass ratios (w/w) in a range of 1/1 to 10/1 to preformed polyplexes at N/P ratios of 2.5–10. The addition of DOSPER liposomes to LPEI polyplexes significantly increased gene transfer (**VI**: Fig. 1). This enhancement was particularly pronounced at N/P ratio 2.5, where the corresponding polyplexes were otherwise ineffective in evoking any transgene expression. Increasing the amount of LPEI in the LPP (N/P ratio) did not

further improve the degree of observed synergism; the transfer efficiency remained almost constant at high DOSPER concentrations and eventually dropped to the levels of polyplex alone at low DOSPER concentrations. It appears that at certain combinations of the vectors and DNA the polyplex is not entirely wrapped by the DOSPER lipid (Song et al., 2012) and the synergism is absent. Transgene expression clearly increased with increasing amount of DOSPER in LPPs (**VI**: Fig 3) and peaked at a DOSPER/DNA ratio of 10. Relative to conventional lipoplexes and polyplexes, the transfection efficiency of LPPs was enhanced (up to 167-fold) and became statistically significant for all LPPs formed at a DOSPER/DNA ratio ≥5 (Table 5.3). At a DOSPER/DNA ratio of 10, where the maximal gene transfer efficiency was achieved, LPPs transfected approximately 20% of cells, which was double the amount achieved with lipoplexes or polyplexes alone (**VI**: Fig. 2).

A similar pattern in transgene expression was observed also for LPPs formed with BPEI (**VI**: Fig. 3). BPEI–LPPs displayed slightly higher transgene expression at an N/P ratio of 5 and slightly lower at an N/P ratio of 2.5 than LPEI–LPPs. In general, both linear and branched forms had comparable transfection efficiencies. Although the transfection activity of LPPs formed at an N/P ratio of 2.5 was clearly higher than the corresponding lipoplexes and polyplexes, the overall transfection levels were nearly the same as for conventional polyplexes at an N/P ratio of 5, especially when BPEI was used (**VI**: Fig. 3).

The comparison of net potentiation of gene transfer between LPPs formed with different-sized PEIs revealed that each PEI has its own specific combinations of N/P and DOSPER/DNA ratio for optimal transfection efficiency (**VI**: Table 1). Although peak synergism was observed at a lower N/P ratio for LPEI than for BEI, the degree of synergism was similar. The synergistic effects on gene delivery observed in this study were, however, weaker than those seen earlier for smaller sized PEIs (Lampela et al., 2002).

Table 5.3 *Enhancement factors of transfection efficiency of PEI/DOSPER lipopolyplexes.*

		DOSPER/DNA ratio (w/w)				
		1	2.5	5	7.5	10
		LPP versus corresponding lipoplex				
N/P 2.5	LPEI	66.2	62.2	7.5	4.0	3.4
	BPEI	27.7	53.3	3.7	2.4	2.2
N/P 5	LPEI	167.0	66.9	9.7	9.2	7.1
	BPEI	10.7	58.9	15.6	13.8	9.6
		*LPP versus corresponding polyplex**				
N/P 5	LPEI	0.8	0.97	1.8	3.3	3.6
	BPEI	0.04	0.7	2.5	4.2	4.2

* Values for N/P 2.5 are not available, as polyplexes formed at this ratio were inactive when used alone. The ratios of transfection efficiencies were calculated from the values in VI: Fig. 3.

6 Discussion

6.1 DNA binding and complexation by cationic polymer vectors

DNA binding and complexation by cationic polymers is the very first step in the transfection process, significantly affecting subsequent delivery steps and final transfection outcome. Too weak binding at the early stages of the transfection might be insufficient to protect DNA against extra- and intracellular nuclease degradation, whereas too tight binding at the late stages might slow down or prevent DNA release from the polymer and hinder the transcription (Schaffer et al., 2000). As the delicate balance between DNA condensation and release is related to the polymer–DNA binding affinity, better understanding of binding mechanisms would be beneficial in designing more efficient delivery vectors.

This study investigated DNA binding properties of the well-known cationic polymer vectors PEIs, PLL and PBAEs. They share a common feature of a cationic amine functional group, but differ in amine valences. Branched PEI contains primary, secondary and tertiary amines, whereas linear PEI has only secondary amines. PLL has a linear topology and only primary amines at lysine side chains are available for DNA binding. Structurally unique PBAEs were chosen from a library of second-generation polymers (Anderson et al., 2005), which represent structures without end-modification, thus having no primary amines. As the selected PBAEs were synthesized with an excess of amine monomers, resulting in amine monomer-terminated polymers, they may contain some secondary amines near the polymer ends. However, the tertiary amines of the polymer backbone are a major component of the PBAEs.

The polymer–DNA binding in this study was determined by a time-resolved fluorescence spectroscopy technique. Compared with steady-state fluorescence analyses, which are commonly used for probing DNA-condensation capability of delivery vectors (Ruponen et al., 1999, Prevette et al., 2010, Malloggi et al., 2015), this analytical approach allows the state of DNA within polyplexes (i.e. relaxed or condensed) to be monitored and gives quantitative information about the DNA binding (i.e. binding constants).

6.1.1 Formation of polyplexes with a core–shell structure

Previously, a core–shell model for positively charged DNA complexes has been proposed in the early works of Kabanov and colleagues (Kabanov and Kabanov, 1995, Vinogradov et al., 1998). According to this model, a hydrophobic core from neutralized polycation/DNA chains formed at polycation excess is surrounded by a hydrophilic shell from charged polycation chains. The fluorescence analysis approach

used in this study provided support for the core–shell structure and further characterized the forming polyplex core and shell.

Polyplex core – At low polymer/DNA ratios (N/P <2), PEI and PLL polyplex formation was characterized by incomplete DNA condensation. Complete DNA condensation occurred at N/P ratio about 2, leading to formation of a neutral polyplex core. At this point, nearly all DNA phosphates were bound by polymer amines, resulting in charge-neutral and unstable particles with a strong tendency to form large aggregates. This N/P ratio is in agreement with other studies observing similar values for complete DNA complexation with BPEI (N/P ~ 1.3 (Clamme et al., 2003), N/P ~ 2.3 (Bertschinger et al., 2006), N/P ~ 2.8 (Boeckle et al., 2004) and N/P ~ 3 (Yue et al., 2011a)) and LPEI (N/P ~ 2 (Perevyazko et al., 2012), N/P ~ 2.6 (Fahrmeir et al., 2007), and N/P ~ 3.5 (Wang et al., 2013)).

Polyplex shell – At a high concentration of polymer (N/P >2), all binding sites in DNA were saturated and excess polymer did not incorporate into the polyplex. The extra polymer formed a shell around the core and significantly increased the polyplex stability against aggregation. Unlike PLL, excess PEI chains in the shell were able to replace PEI chains previously bound in the polyplex core. This finding is in general agreement with previous molecular dynamics simulations (Sun et al., 2012), where added PEIs were shown to replace the PEIs bound to the aggregates formed at low N/Ps. The presence of such core–shell chain exchange makes the PEI polyplex structure clearly more dynamic. Similarly, using a theoretical model, Hou et al. (2011) showed that the steric hindrance or repellence between PEI or DNA molecules prevents PEI and DNA molecules from attaching closely to each other, and thus creating a vacant space in PEI polyplexes, which is presumably occupied by a solvent, and indicates a loose structure. On the other hand, nearly ten times larger molecular weight of PLL in comparison to PEI might lead to increased PLL-DNA interactions, tighter binding, resulting in a more stable core–shell structure.

The exchange of PLL polymer molecules between the core and shell in polyplexes might be hindered by bulkier chain of PLL. For core–shell dynamics, PLL and PEI with different molecular weights were selected regarding their DNA binding and gene delivery efficiencies; smaller 20-kDa PLL is unable to condense DNA at the same N/P ratios as the used 200-kDa PLL and 25-kDa PEI (Ruponen et al., 1999) and forms polyplexes that easily aggregates at physiological salt levels (Ward et al., 2001). Therefore, the difference in molecular weights of polymers used in this study may also partially explain why the core–shell exchange takes place in PEI but not in PLL polyplexes.

According to the results of time-resolved fluorescence, the physical states of PEI polyplexes were not affected by free polymer, and therefore free polymer must have an effect on its own on the cellular level. This is consistent with the recently published results of Cai et al. (2016) and Klauber et al. (2016). They demonstrated that PEI chains bound in the polyplex core play a main role in condensation and protection of nucleic acids during cell uptake and intracellular trafficking, whereas free PEI chains promote gene transfection in the intracellular space, separately from the core polyplexes.

6.1.2 Mechanisms of polymer–DNA binding

Binding mechanisms were determined by independent and cooperative binding models. The cooperative binding model, in contrast to the independent binding model, reflects the influence of one bound amine ligand on the binding affinity of the second polymer amine at unfilled DNA phosphate sites. In both models, the polymer–DNA binding affinity for PEIs and PLL was characterized by the presence of two phases. According to the independent binding model, the binding affinity between polymer and DNA was biphasic, suggesting a change in the binding mechanism with increasing polymer amine concentration; at low concentrations, the polyplex formation was observed and at higher concentrations the reaction had reached completion. The biphasic binding mechanism was observed also with the cooperative binding model. As the cooperative binding model enabled a more accurate description of polyplex behaviour, cooperative binding constants are presented in the following discussion.

PEIs – The mechanism of BPEI polyplex formation changed from independent binding without cooperativity to positive cooperative binding at an N/P ratio close to 0.6. At this ratio, there is still an excess of DNA, and therefore, it could be speculated that the change in the binding mechanism reflects the formation of locally charge-neutral, hydrophobic regions of polymer-bound DNA that facilitate the further binding of cationic polymer to DNA, as proposed by Parker et al. (2002). Interestingly, at practically the same N/P ratio of 0.68, a partial DNA complexation and formation of primary PEI complexes were observed by scanning force microscopy (Perevyazko et al., 2012). At high polymer concentrations (N/P ~2), the positive cooperativity changed to the negative, suggesting that further PEI binding was hindered by the already bound amines. At this point, the binding isotherm reached its maximum and the polyplex core formation was completed. Similar biphasic behaviour was observed also for LPEI.

In contrast to BPEI, LPEI polyplex formation at low polymer concentrations was characterized by binding with negative cooperativity, leading to a smaller overall binding constant, which corresponds to a weaker binding. The difference in DNA binding could be attributed to different PEI molecular architecture. The linear structure of LPEI in which all amines are located in the polymer backbone could cause steric hindrance during the binding. This is consistent with the findings of molecular dynamic simulations reported by Sun et al. (2012), where the different PEI branching resulted in distinct binding patterns. In their study, BPEI was observed to adhere to the DNA surface like beads, whereas LPEI adhered like cords, thus covering DNA surface more efficiently. The better surface coverage, however, limited the number of LPEI molecules that could complex with DNA. The difference between LPEI and BPEI condensation behaviour was also observed experimentally in a study using dynamic laser light scattering (Dai and Wu, 2012). The authors proposed that LPEI chains can align themselves on each DNA chain, whereas BPEI chains can bind two segments on different DNA chains and cross-link them, leading to more compact polyplexes. In this study, the negative cooperativity of LPEI changed to positive with increasing polymer concentration. It is likely that the increasing number of LPEI chains present in solution reduced the chance of perfect alignment on DNA, thus

creating more space for other LPEI chains on the DNA surface, as seen by Dai and Wu (2012).

Both overall binding constant and average binding constant per amine were clearly smaller for LPEI. The weaker DNA binding (i.e. smaller binding constant) of LPEI is in line with a common finding that LPEI polyplexes are more easily disrupted by various strong polyanions (Bertschinger et al., 2006, Hahn et al., 2010, Dai et al., 2011, Kwok and Hart, 2011, Dai and Wu, 2012) than their BPEI counterparts. In addition to better resistance of BPEI polyplexes upon exposure to polyanions, more effective DNA binding can be observed in formation of smaller and denser particles in our study and in the literature (Dunlap et al., 1997, Itaka et al., 2004, Intra and Salem, 2008).

PLL – Similar to BPEI, the mechanism of PLL polyplex formation changed from independent binding to cooperative binding close to an N/P ratio of 0.6. Unlike BPEI, PLL displayed a clearly lower degree of cooperativity, leading to a smaller overall binding constant. The distinct values of overall binding constants of PLL and BPEI are likely to be due to differences in their molecular structure. While BPEI active amine groups are situated along the polymer backbone spaced only by two carbon atoms, PLL active amines are located on side chains and separated from the backbone by four carbon atoms. Therefore, it seems that large hydrocarbon skeleton of PLL sterically hinders the access of some of its amines to DNA phosphates. This in turn reduces the degree of cooperativity and weakens the binding to DNA. This assumption is further supported by molecular dynamics simulations by Ziebarth and Wang (2009), where the relatively smaller and more densely charged PEI molecule was shown to occupy less space on the DNA and condense DNA to a greater extent than the long linear PLL chains.

PBAEs – The mechanism of PBAE polyplex formation at low polymer concentrations was characterized by negative cooperativity, leading to small overall binding constants. The cause for the negative cooperativity probably originates from the steric repulsion between PBAE molecules due to many electronegative groups on their backbone and side chains. As a result of negative cooperativity, PBAEs saturated always below 100%, while PEIs and PLL saturated near 100%. Relative to BPEI, the overall binding constants were extremely low. One explanation might be that PBAEs have relatively low amine densities compared with BPEI. Therefore, the distance between amines is so long that the cooperativity of binding to DNA is lower than for BPEI. PBAEs also lack primary amines and have a lower ratio of secondary to tertiary amines compared with BPEI. As a consequence, PBAEs require more amine groups than BPEI in order to fully complex DNA. Typically, very high N/P ratios of 50 are needed for efficient transfection with PBAE polyplexes relative to N/P 6–12 with BPEI (Anderson et al., 2005, Green et al., 2006). When compared to BPEI, such very high N/P ratios do not cause serious problems with cytotoxicity due to PBAEs rapid degradation.

With increasing polymer concentrations, the binding between DNA and PBAEs was unchanged (C28, C36, AA24) or changed at an N/P ratio of about 27 to a binding characterized by (i) less negative cooperativity (F28, AA28, C32), (ii) nearly independent binding (E28, JJ28), and (iii) moderate positive cooperativity (D24, U28).

It is possible that the change in the binding mechanism at such a high N/P ratio is linked to the formation of polyplex core, as proposed by Ketola (2014).

6.1.3 Environmental pH and its influence on polymer–DNA binding

Polyplexes are usually prepared in physiological pH buffers or cell culture media in order to be identical to the cell culture environment. However, the pH of a medium used for complexation plays an important role in polymer–DNA binding affinity, as amine groups of cationic polymers change their degree of protonation depending on the pH of the environment. For example, BPEI has a considerably higher protonation ratio of amine groups at low pH; 45% of amines are charged at pH 5 and only 20% at pH 7 (Suh et al., 1994).

This study showed that the environmental pH had a clear effect on the mechanism of polyplex formation. With a decrease in pH, the biphasic binding associated with independent and positive cooperativity phases changed to a single positive cooperative mechanism with an apparently higher degree of cooperativity. The binding affinity between polymer and DNA increased as the pH of complexation media decreased, reflecting the higher protonation of the polymer. This finding is consistent with earlier isothermal titration calorimetry experiments that observed an increase in the binding affinity of 600 Da BPEI to the DNA at lower pH (Utsuno and Uludag, 2010). Similarly, in molecular dynamics simulations, PEIs with a higher protonation ratio demonstrated stronger binding to DNA due to closer contacts of PEI amines with DNA phosphates and formation of more stable and compact polyplexes (Sun et al., 2011, Bagai et al., 2013).

The pH-dependent DNA binding affinity was more pronounced in PEI than PLL due to differences in amine group content. Unlike PLL, whose primary amines are completely charged at any pH below 9, PEI charge density increases when pH of media acidifies from 7.4 to 5.2 due to additional ionization of secondary amine groups. This effect was larger for BPEI than LPEI because of additional tertiary amine groups present in BPEI. Therefore, especially for polymers containing secondary and tertiary amines, it appears more beneficial to prepare polyplexes at a lower pH, although the transfection is performed at biological pH. This assumption is in good agreement with Fukumoto et al. (2010), who demonstrated experimentally that, compared with pH 7.4, formation of LPEI polyplexes at pH 4 leads to higher transfection efficiency.

As the binding affinities were pH-dependent, it could be inferred that, in addition to polyplex formation in a test tube, the change in the environmental pH could also have an effect on polyplexes within cells. The higher positive cooperativity and DNA binding affinity of polymers at low pH could lead to a greater tightness and stability of polyplexes in acidic endocytic vesicles, while the lower affinity to DNA at neutral pH could contribute to polyplex loosening and DNA release in the cytosol. The quantitative DNA binding analysis at two different pH values obtained in this study supports Kang et al. (2012), who showed that in acidic medium, BPEI and PLL polyplexes may prevent or delay disassembly compared with neutral pH of 7.4.

6.2 Relationship between DNA binding affinity and transfection efficiency of cationic polymers

According to this study, no direct correlation existed between overall binding constants of cationic polymers and their transfection efficiencies. The overall binding constants, ranked in descending order, were as follows: BPEI, LPEI, PLL, and PBAEs (D24, U28, JJ28, E28, AA28, C32, F28, AA24, C28, C36), whereas transfection efficiencies decreased in a completely different order: PBAEs (C32, JJ28, C28, U28, AA28, AA24, D24, F28, C36, E28), LPEI, BPEI, and PLL. The results strongly indicate that the overall binding constant alone describes only the first step in the transfection process – i.e. polyplex formation. For this reason, the value of the binding constant cannot fully predict the outcome of transfection, as the transfection efficiency is also influenced by how successfully a particular polymer manages to overcome the intracellular delivery barriers described in Figure 2.1. However, one of the crucial steps for efficient gene delivery is the release of DNA from the nanoparticle to the cytosol. Since this process is the reverse reaction of the nanoparticle formation, the binding constants could provide useful information on how easily polyplexes dissociate in the cytoplasm. Indeed, it was suggested recently that DNA release from polyplexes appears to be inversely proportional to the binding affinity between DNA and PBAEs (Bishop et al., 2013).

PLL – For PLL, there was an association between low overall binding constant and low transfection efficiency. This might have resulted from the fact that the linear structure of PLL with only primary amines affects both polyplex formation and intracellular delivery in a similar way. Despite the fact that the large hydrocarbon skeleton may weaken binding to DNA during polyplex formation, PLL still forms stable polyplexes that were shown to enter cells with a similar efficiency as BPEI polyplexes (Männistö et al., 2005). PLL–DNA binding relies almost exclusively on primary amines that are fully charged at physiological conditions, whereas about only half of all amines are protonated in BPEI (Tang and Szoka, 1997).

As can be seen from the higher average binding constant per amine group, the primary amines of PLL bind with DNA more strongly than the secondary amines of LPEI, tertiary amines of PBAEs, and the combined affinity between primary, secondary, and tertiary amines of BPEI. This strong binding is consistent with the finding that PLL polyplexes, in contrast to PEI polyplexes, are more resistant to disruption following incubation with competing polyanions (Ruponen et al., 2001, Itaka et al., 2004). Within the cells, however, such high binding affinity of primary amines can be a considerable disadvantage, as it may hinder the release of DNA from the vectors and lower the gene transcription. Nevertheless, the difference between the average binding constants per amine of PLL and BPEI was relatively small (only 20%) and could not solely explain the marked difference in DNA release and gene transfection efficiency between PLL and BPEI polyplexes.

The poor transfection performance of PLL is also related to the difference seen in structural dynamics between PLL and BPEI. While PLL polyplexes had a rigid core–shell structure, BPEI polyplexes were capable of exchanging PEI molecules between the core and shell. Such mobility of PEI molecules makes the polyplexes more

dynamic, and hence, more sensitive to dissociation and DNA release at the cellular level, leading to active transfection. Furthermore, the presence of fully charged primary amines lowers the endosomal buffering capacity of PLL (Sonawane et al., 2003), which in turn limits the polyplex escape from endosomal/lysosomal digestion and contributes to a very poor transfection efficiency.

PEIs – Although the overall binding constant of BPEI was clearly the highest, BPEI displayed much lower transfection efficacy than PBAEs or LPEI. One of the factors causing the lower transfection efficacy could be a less efficient intracellular release of strongly bound DNA. It has been shown that the strong binding makes the DNA less accessible to the transcription machinery than loosely bound LPEI polyplexes (Itaka et al., 2004). Another factor could be related to BPEI toxicity and non-degradability. Compared with BPEI, less toxic PBAEs can be used at higher N/P ratios to enable nanoparticle concentrations with 5-fold higher total buffering capacity (Sunshine et al., 2012), and on longer time scales, the degradation of PBAEs in the cells may trigger a release of DNA inside the cells. Also, the overall binding constant values describe only the formation of the nanoparticle core, that is, until all the phosphate groups on the DNA are bound at an N/P ratio of about 2. After this, excess polymer binds to the nanoparticle core, forming a protective shell around it. This excess of polymer has been shown to be a crucial factor in getting DNA to its destination, into the nucleus of cells (Boeckle et al., 2004).

PBAEs – Within the series of tested PBAEs, there was no straightforward correlation between the overall binding constants and the transfection efficiencies, as polymers with comparable DNA binding affinities displayed completely different transfection efficiencies. Interestingly, some similarities between overall binding constant and transfection efficiency could be observed for PBAEs that differed only in the length of their monomers. Changing the monomer structure just by one carbon atom led to changes in overall binding constants and transfection efficiencies that followed the same trend.

Unlike binding constants, the nanoparticle formation efficiency was positively correlated with increased transfection efficacy for eight out of ten PBAE polymers. This demonstrates the usefulness of the developed fluorescence method in DNA complexation studies within polymer libraries for the prediction of transfection efficacy. Thus, DNA binding affinity and nanoparticle formation efficiency are important parameters for effective non-viral polymeric gene delivery and these parameters can be specifically designed by tuning polymer structure.

6.3 Role of free polymer in polyplex-mediated delivery

The most efficient polyplex-mediated delivery of DNA has been routinely observed with polyplexes prepared at relatively high N/P ratios, i.e. with a large excess of polycation. Cationic polymers, however, bind DNA only up to a certain (lower) N/P ratio and, beyond that point, any additional polymer remains free. Although the free

polymer stays free or weakly associates with the polyplexes in solution, it plays an essential role in transfection studies.

The excess of free polymer is easy to use in vitro, but it is questionable whether it is beneficial also in vivo due to rapid dilution in the systemic circulation and pronounced toxicity (Boeckle et al., 2004, Fahrmeir et al., 2007). At cellular level, however, the excess polymer has been shown to significantly improve delivery by both DNA- and siRNA-containing PEI polyplexes (Yue et al., 2011a, Klauber et al., 2016). The fact that free polymer promotes gene transfection even when added to cells later than polyplexes suggests that free polymer chains may aid mostly with endosomal release and later steps of delivery (Boeckle et al., 2004, Thibault et al., 2011). It should be noted, however, that the detailed mechanism by which the free cationic polymer facilitates transfection remains unclear.

The surplus of polymer gives polyplexes a positive surface charge that stabilizes polyplex particles against aggregation and increases affinity for negatively charged cell membranes (Haensler and Szoka Jr, 1993). On the other hand, positively charged polyplexes will also readily interact with highly anionic cell-surface GAGs. Such GAG-polyplex binding is one of the barriers to efficient gene delivery (Ruponen et al., 2004). The current study, therefore, focused on the role of free polymer in the interactions between polyplexes and cell-surface GAGs.

For this part of the study, we selected a branched PEI as a model carrier and separated free PEI chains from PEI polyplexes using size exclusion chromatography, which enables preparation of stable and positively charged polyplexes without free PEI. Such purified polyplexes had similar size and surface charge characteristics as their parent polyplexes, ensuring the same electrostatic interactions with cell-surface GAGs. This is in contrast to a direct mixing of PEI with DNA at an N/P ratio of 3, used in the studies of Wu and colleagues (Yue et al., 2011a, Yue et al., 2011b, Cai et al., 2016), which resulted in particles with a near neutral surface charge (Dai et al., 2011). Our study indicated that free PEI as such is not a key factor in efficient transfection. Rather, the inhibition of polyplex transfection by cell-surface GAGs is the critical factor hindering PEI-mediated delivery. Free PEI, however, significantly helps to decrease the inhibitory polyplex interactions with cell-surface GAGs and its role is linked to the amount of GAGs in the target cell type. On the contrary, Kichler et al. (2005) excluded the role of cell-surface GAGs in the free PEI effect, but their study was incomplete since it did not include purified DNA complexes. The current results clearly showed the interplay between free PEI and cell-surface GAGs. Based on the results, a hypothetical picture describing mechanisms of a positive effect of free PEI on transfection can be drawn. The improvement in transfection may be due to one or all of the following possibilities:

Polyplex integrity – GAGs can bind to polyplex and interfere with polyplex stability, resulting in a premature release of DNA from the polyplex and a decrease in transfection efficiency (Ruponen et al., 2001, Ruponen et al., 2004). Since polyplexes and free PEI were in dynamic equilibrium with continuous PEI exchange between the core and shell, it is possible that polyplexes without free PEI are disturbed or loosen with cell-surface GAGs more easily than polyplexes with an excess of PEI. Free cationic PEI may bind to anionic GAGs, and thus, reduce the undesirable

GAG-binding and subsequent dissociation of polyplexes by GAGs even before entering the cells. This was also seen in a simple in vitro assay where the presence of excess PEI increased the resistance of polyplexes to heparin-induced displacement (Bertschinger et al., 2006). On the other hand, the excess of PEI may have a detrimental effect in later stages of transfection, as it can disturb the release of DNA from the carrier and indirectly reduce the level of transcription (Matsumoto et al., 2009). However, a very interesting observation recently reported by Wu and colleagues (Cai et al., 2016) was that free PEI actually enhances transcription efficiency by 4-fold. The question then arises to what extent is the difference seen in transcription efficiency the consequence of polyplex preparation conditions. As already noted, their polyplexes without free PEI were generated directly at an N/P ratio of 3. Since the polyplex formation at such a low N/P ratio is known to result in large polydisperse aggregates, as seen in our results and those of Boeckle et al. (2004), it is probable that the polyplex preparation conditions used in Cai et al. (2016) also resulted in aggregation. The polyplex aggregation has been demonstrated to interfere with the efficient release of pDNA (Bertschinger et al., 2006) and may thus explain reduced transcription efficiency in the absence of free PEI.

Cellular uptake – PEI polyplexes were shown to electrostatically interact with polyanionic GAGs and compete with free PEI for cellular association in a manner similar to receptor competition (Boeckle et al., 2004, Bausinger et al., 2006). At this stage, the free PEI probably competes with polyplexes for binding sites at the cell surface, thereby decreasing cellular uptake, although it still improves transfection. Similarly, no positive effect of free PEI on cellular uptake was seen in Boeckle et al. (2004), Dai et al. (2011), and Klauber et al. (2016). Combining two important observations that free PEI decreased the quantity of cellular pDNA (the current study) and enhanced the transfection efficiency of already internalized polyplexes (Boeckle et al., 2004, Dai et al., 2011) suggests that free PEI has only a minor role during the cellular uptake of polyplexes and its positive effect on transfection efficiency is mediated mainly via intracellular mechanisms.

Endosomal escape – It is known that, upon internalization, PEI polyplexes inside cells are associated with GAGs and co-localize within the same endosomal vesicles (Payne et al., 2007). In the endosomes, the GAG-polyplex binding may strongly reduce proton-absorbing capacity of PEI chains in the polyplex core (Won et al., 2009). It is also likely that GAG-bound PEI may not swell in endosomes as efficiently as the free PEI (Bieber et al., 2002), and thus, cannot contribute to endosomal escape of the polyplexes. Free PEI in such a situation would provide an extra buffering capacity to the endosomes and facilitate the escape of polyplexes from endosomes via a proton sponge effect or by binding and intercalating into inner surface of the endosomal membrane (Vaidyanathan et al., 2016).

Intracellular distribution – Free PEI has an ability to bind and saturate anionic membrane proteins on cell surfaces (Bausinger et al., 2006) and even induce long-lasting cell membrane permeability (Vaidyanathan et al., 2015). Therefore, it may potentially facilitate alternate routes of polyplex internalization and modify intracellular distribution through this interaction. By binding to cell-surface GAGs, free PEI may partially prevent polyplexes from being trapped by heparan sulfates and

following the unfavourable GAG-mediated pathways that shuttle polyplexes to lysosomes, where they may be enzymatically degraded. The study by Cai et al. (2016) argues in favour of the hypothesis that free PEI helps polyplexes to avoid lysosomes in the endolysosomal pathway. Their work showed that free PEI reduced the lysosomal entrapment of internalized polyplexes, presumably by adsorbing on the inner cell membrane and interfering with SNARE signal proteins, so that the inter-vesicular endosome-lysosome fusion between lysosomes and polyplex-containing vesicles is slowed down or disrupted. They determined that the presence of free PEI helps to divert more than half of the polyplexes that would otherwise have been directed to the lysosomal degradation pathway. Moreover, longer and branched free PEI chains were more effective in preventing lysosomal entrapment than short and linear ones, presumably by cross-binding multiple signal proteins on the cell membranes more efficiently (Klauber et al., 2016). In an extension of this hypothesis, Thibault et al. (2011) suggested that the excess of polycation could provide additional protection during endosomal maturation by competing with polyplexes as a substrate for lysosomal enzymes.

Due to the complexity of the cell–polyplex interactions, only a few possible mechanisms involved in the complicated interactions between free PEI, polyplexes, and cell-surface GAGs are discussed. The combined data from time-resolved fluorescence spectroscopy, core–shell dynamic studies, transgene expression, and cellular elimination demonstrate that the beneficial effect of free PEI is mediated at the intracellular level via interactions with GAGs. The free PEI–GAGs interactions lead to alternation in gene transfer by various mechanisms and are the key factor explaining why PEI polyplexes require an excess of free carrier for effective transfections. It is likely that the same mechanistic interpretation has implications also for other non-viral vectors containing secondary and tertiary amines. This study contributes to the limited data on free polycation in the current literature.

6.4 Role of PEI structure in nucleic acid delivery via cationic lipopolyplexes

Lipopolyplexes represent a gene delivery system that combines favourable properties of cationic polymers and liposomes in a single nanoparticle. These two components have been shown to cooperate in the transfection process, and hence, to enhance transfection efficiency. This synergistic enhancement was observed for a wide range of lipids and polycations (Rezaee et al., 2016). The level of synergism varies depending on delivery vectors used for LPP formation (Lampela et al., 2004), but little is known about how the polymer structure affects the synergy observed in transgene expression. In this study, the effect of PEI structure on LPP-mediated synergism was investigated.

Various PEI–LPPs for delivery of DNA have been reported to form core–shell structures consisting of a polyplex core and a lipid shell (Matsumoto et al., 2008, Schäfer et al., 2010, Song et al., 2012). For these systems, the best synergy was

achieved when the polyplex core was condensed at relatively low polymer/DNA ratios and combined with a moderate amount of lipid. Such behaviour was also seen in this study where the synergistic effect on the transfection efficiency was particularly pronounced at low N/P ratios, at which the conventional polyplex and lipoplex alone displayed minimal activity. The difference in efficiency could be attributed to the improved stability of LPP delivery complexes compared with the polyplex equivalents. At N/P 2.5, there is little excess PEI, and nanoparticle formation is accompanied by immediate aggregation. The addition of DOSPER to polyplexes pre-condensed at a low N/P ratio further condenses DNA to nearly the same levels as for polyplexes prepared at high N/Ps (Lampela et al., 2003). Therefore, the additional cationic lipid component could increase cationic charges and offer greater protection to the relaxed polyplex core, improving transfection outcome. Although Song et al. (2012) suggested a loosely packed polyplex core as being the most beneficial in transfection, in our study LPPs with a more tightly packed polyplex core formed at N/P 5 showed better ability to transfect cells. While the initial polyplex core is very tightly packed, the addition of cationic lipid could interfere with polymer–DNA interactions and weaken the polymer–DNA binding in LPPs as seen in Munye et al. (2015), making DNA more accessible for transcription and possibly contributing to enhanced gene expression.

In general, the effect of PEI branching on the PEI/DOSPER-mediated synergism was minimal. Both linear and branched PEI–LPPs showed comparable synergistic enhancement in transgene expression. This is in contrast to other published works on LPPs where either BPEI (Pelisek et al., 2006) or LPEI (Tros De Ilarduya et al., 2010) formulations were reported as being the most efficient. This discrepancy can be explained by the different compositions of media used for complexation, leading to different particle size and inherent kinetic stability under salt conditions. The size of LPEI polyplexes is strongly influenced by the salt concentration during preparation, unlike the size of BPEI polyplexes (Wightman et al., 2001). Similarly BPEI–LPPs displayed better resistance to aggregation than LPEI–LPPs (Pelisek et al., 2006). Furthermore, it is important to note that the level of synergism of PEI/lipid-mediated transfection usually varies between cell lines and cationic lipids used to form LPPs (Lampela, 2003), suggesting that whatever the mechanism of synergy may be the composition of individual components must be specifically optimized for each transfection model. However, the potentiation for high molecular weight PEIs found in this study was clearly weaker than that for low molecular weight PEIs obtained previously (Lampela et al., 2002). It is evident that the enhancement of PEI/liposome-mediated gene delivery by different PEI species is a common attribute that is more associated with PEI size than PEI structure.

PEI–LPP formulations provide a simple and inexpensive method for enhancing non-viral gene delivery without the need of using excessive amounts of cationic carriers. An important objective for future studies is to determine detailed structure–function relationships of the components used in combination to shed light on the underlying mechanism of the synergism.

7 Conclusions

In this thesis, transfection studies and fluorescence spectroscopy were combined to gain a better mechanistic understanding of polyplex formation and to clarify the role of free cationic polymers in non-viral gene delivery. The main conclusions are as follows:

A general finding for different polymers is that the mechanism of polyplex formation changed with an increasing amount of polymer (N/P ratio) in the forming polyplex. In addition, minor changes in polymer structure induced different binding mechanisms. For PEI and PLL, the binding mechanism was associated with non-cooperative and positive-cooperativity phases. The polyplex core formation was completed at N/P ~2, and beyond this point a shell of excess polymer was formed around the polyplex core. Unlike PLL, PEI molecules were able to undergo exchange between the core and the shell of polyplexes. This exchange makes PEI polyplexes a more dynamic system and may partly explain the different DNA release and transfection efficiency between PEI and PLL.

The environmental pH during polyplex preparation strongly influences the mechanism of polyplex formation. By lowering the pH of the complexation medium, the binding affinity between polymer and DNA increases and is characterized by a single positive cooperative phase. This suggests that it may be beneficial to prepare polyplexes containing secondary and tertiary amines at lower pH, although transfections are performed at biological pH.

No direct correlation exists between the overall binding constants and transfection efficiency. This is presumably because the value of the binding constant describes only the polyplex formation and does not therefore reliably predict the final outcome of transfection, which is also strongly affected by various intracellular factors. Unlike overall binding constants, the relative efficiency of nanoparticle formation positively correlated with the transfection efficiency. The presence of such a correlation for different vector molecular structures gives further insights into the mechanisms of polyplex-mediated gene delivery. Time-resolved fluorescence spectroscopy used in this study represents a useful method to investigate delivery vector–DNA interactions and binding quantitatively.

The excess of free vector can be considered a highly important element in polyplex-mediated gene delivery. Free PEI has no influence on the physical state of polyplexes, suggesting that it may have an effect of its own on the cellular level. Its beneficial effect was observed in all cell lines studied with the notable exception of cells lacking cell-surface GAGs. At the cell surface, free PEI interacts with ubiquitous GAGs and these interactions lead to alterations in gene transfer, mainly involving intracellular delivery steps. The advantage of using free PEI in polyplex formulation is that free PEI helps to decrease the inhibitory polyplex interaction with GAGs, ensuring efficient delivery.

PEI/DOSPER-mediated synergism in transfection efficiency is a common attribute of different PEI species regardless of their size or structure. The potentiation of transfection efficiency was, however, higher for PEIs with lower molecular weights.

8 Future prospects

Gene therapy research has been evolving very rapidly over the last three decades. It has moved from small-scale academic studies to first-generation gene-based medicines. However, despite the invested time and efforts, the European Medicines Agency (EMA) has approved only two gene therapy products, and the U.S. Food and Drug Administration (FDA) has yet to approve any gene therapy treatments at the time of writing this thesis. These novel biopharmaceuticals are based on viruses, and thus, genuine issues concerning their safety, manufacturing and regulatory approval render them demanding and expensive products (Wirth et al., 2013). For example, the cost of more than one million euros per patient (Orkin and Reilly, 2016) makes Glybera, the first gene therapy product, the world's most expensive medicine with a questionable long-term efficacy (Bryant et al., 2013).

Although there are currently no commercially approved non-viral gene therapy products and the number of completed clinical trials is limited, non-viral gene delivery vectors have significant potential to become next-generation delivery systems of nucleic acids because of their flexibility, greater simplicity of manufacturing, and low host immunogenicity. Non-viral vectors, especially polymeric vectors, are in a rapid phase of development. They have evolved from simple off-the-shelf polymers into precise, sequence-defined polymers with targeted and dynamically responsive delivery (Wagner, 2012). However, the main limitation of current polymeric gene delivery vectors is their relatively low gene transfer efficiency. Therefore, the biggest challenge for gene delivery scientists is not only to engineer new improved vectors but also to better understand which polymer structural features enable effective nanoparticle formation and delivery and how they help overcome cellular delivery barriers. This challenging task is further complicated by the fact that these features are not versatile, as different types of cargos (pDNA or RNA) require different vectors and the delivery mechanisms are dependent on cell type (Bishop et al., 2015).

It is very difficult to construct a full mechanistic understanding based on the existing literature because such relationships are poorly addressed and existing data are fragmented (Malloggi et al., 2015). Poor understanding of structure–function and structure–activity relationships of polymeric vectors hinders the development of more sophisticated vectors. The non-viral gene delivery field would benefit greatly if more attention is paid to correlating polymer structure, nucleic acid binding affinity, and biological efficacy. The development of more accurate techniques, such as surface plasmon resonance for monitoring polyplex cell uptake, and fluorescence lifetime microscopy for monitoring formation/release processes of polyplexes inside cells, is underway. The knowledge of quantitative data on how different changes in polymer structure are related to gene expression efficacy will be beneficial for constructing a predictive quantitative structure–activity relationship (QSAR) models for each step of polyplex-mediated gene delivery.

Moreover, the transfection efficiency of polyplexes is critically dependent on the presence of the free polymer fraction. In this case, gene delivery research needs to fully elucidate the interplay between free polymers in polyplexes and cell surfaces, or

even subcellular organelles. However, current understanding of the cell–polyplex interaction is insufficient and is considerably slowing down progress towards more efficacious vectors (Klauber et al., 2016). More detailed research addressing the role of free polymer in cell-surface GAG–polyplex interactions is therefore needed. The key to success will be finding new GAG-labelling approaches that allow more precise visualization of the dynamics of free polymers, polyplexes, and GAGs in living cells (Favretto et al., 2014). A future challenge will be to determine whether it is possible to control the free polymer fraction for systemic administration so that it is of benefit also for in vivo delivery.

Despite rapid progress over the past decade, the development of gene medicines based on polymeric vectors is still in the beginning and requires further improvements in order to enhance transfection efficacy. While it is likely to be successful in the future, it will take number of years before non-viral vectors surpass their viral counterparts and become a part of a standard therapy. The therapeutic potential of these systems is enormous, and already few polyplex-based delivery systems have been used experimentally in clinical studies with a promise to bring relief to patients with cancer. Future studies will certainly bring more detailed understanding of the mechanisms behind nanoparticle formation and gene delivery, and thus facilitating rational and mechanism-based design of polymeric vectors.

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